Human Papilloma Virus and Oral cancers: sexual behaviour as a risk factor.

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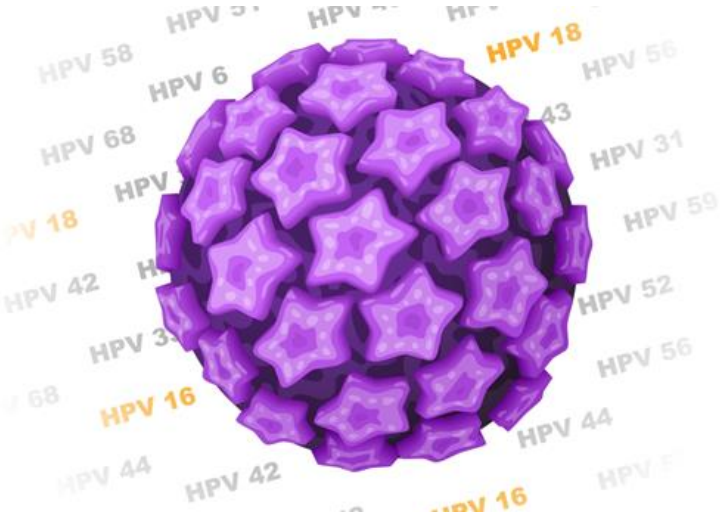
The author confirms there were no conflicts of interest in this thesis.



Dissertation submitted on, 27th March 2017, in Partial Fulfilment of the Requirements for the Doctorate in Health Care Science (DHSci) at De-Montfort University.

HPV – known cause of cervical cancer, Increasing HNSCC incidence, change in sexual habits.

Abstract

AIM & OBJECTIVES: Human papilloma virus (HPV) has been related to cervical infection, however, its part in Head and Neck Squamous Cell Carcinoma (HNSCC) is still debatable and is easy to refute. Suspicion of HPV causation is heightened when carcinomas arise in patients that are young and have never smoked. The present UK based study undertaken at Northampton NHS Trust endeavoured to determine the extent to which HPV is an entity in HNSCC in the UK. Furthermore, the study investigated whether sexual behaviour (as measured by sexual health clinic (SHC) attendance) is linked the acquisition of HPV associated HNSCC in young age groups. HNSCC incidences and sexual trends in the UK were collected from publicly available databases to identify if there were any changes at a national level in sexual behaviours and their influence on HNSCC in young age groups.

MATERIALS & METHODS: PCR was used to evaluate the presence of HPV in biopsy samples from of 99 patients diagnosed with HNSCC at Northampton Hospital from 2006 to 2014. Patient demographics on age, sex, smoking, alcohol use and SHC attendance were also collected. All HPV PCR positive biopsies were further genotyped using an ABI 3130xl genetic analyser. Databases in the UK; including GLOBOCAN, NATSAL and PHE were searched for data on HNSCC prevalence, sexual behaviour trends and vaccine uptake. Multinomial regression explored the relationship between HPV positivity and sex, age, smoking, drinking, race and SHC attendance.

RESULTS: PCR showed that 25.2% (25/99) of biopsies tested were positive for HPV and were all obtained from white participants. Most specimens (23, 92%) were high-risk (HR) HPV 16 positive with a mean age of 56 for HPV positivity and 72% of the cases 50-60 years old. Smokers were 11% in total (11/99) with most 88.9% participants (88/99) being non-smokers. HPV positivity was strongly linked with non-smoking history ($p<0.001$); no alcohol abuse ($p<0.001$); male gender ($p<0.001$); young age less than 60 years ($p<0.001$) and SHC attendance ($p<0.001$). A Kruskal-Wallis *post hoc* test affirmed the impact of age on HPV positivity ($p=<0.05$).

GLOBOCAN and Cancer Research demonstrated a rising UK HNSCC pattern of over 200% for both sexes from 1975 to 2011. The three NATSAL surveys undertaken in 1990-1991, 1999-2001 and 2010-2012 demonstrated an overall increase in opposite and same sex partners. The UK average of individuals engaging in oral sex was in the younger age groups of between 16 and 54 with at least 70% of males and 63% females of that age engaging in oral sex. Finally, NASTAL 1, 2 and 3 surveys reported 20 vs 15; 25 vs 55; 55 vs 65 of males and females respectively with more than 10 sexual partners to have attended the SHC. The UK immunization take-up was over 90% countrywide.

CONCLUSION: Few research studies have been conducted to date on HPV as a cause of HNSCC in the UK. The present research showed 25.2% of HNSCC to be caused by HPV, with the high risk (HR) genotype 16 (the leading cause of cervical cancer) accounting for 92% (23/25) of the cases. These outcomes affirmed the high prevalence of HR-HPV in HNSCC, with a rate of 25.2% similar

to those reported previously. Routine HPV testing in those aged below 60 is therefore warranted. Smoking and drinking showed negative correlation; the young age of below 60 and attendance of the SHC for both sexes showed a positive correlation with HPV positive HNSCC. NATSAL data showed increased sexually risky behaviour coupled with attending the SHC in younger ages for both sexes. Increased sexually risky behaviour as shown in NASTAL surveys may be the reason why young age and SHC attendance is positively correlated with HPV HNSCC. The study highlights a conceivable relationship between HPV positive HNSCC in those under 60 years with no smoking history who attended the SHC. Smoking and drinking are known risks for HNSCC in those past 65 years of age; the negative association with HPV HNSCC in the young in the present research revealed smoking and drinking to have reduced association with HPV HNSCC. The reported HR-HPV positive HNSCC in young age groups inform future vaccination strategies and consequently decrease the quantity of HPV HNSCC's.

PUBLICATIONS: Abstract accepted, Research presented, Poster presented at the School of Allied Health Sciences Research Conference (SAHRC) 2016 – De-Montfort university.

Acknowledgements

I would like to express my sincere gratitude to all who have made it possible for me to undertake this research, special thanks to:

My supervisor Professor Richard Jenkins for his support for this research through all the difficulties, his support, patience and encouragement are appreciated. My supervisor Dr. Graham Basten, for his support and encouragement throughout the course of this research. My supervisor Dr. Parvez Harris, for his support and encouragement throughout the course of this research.

Mr Augustus Lusack, who was also was my supervisor at Northampton Hospital for his support and encouragement and sourcing out funding for this research as Director of Pathology, I would not have done it without your support.

The Histopathology department at Northampton Hospital for their support in the research and providing biopsy samples used for the present research and contribution towards data collection, special thanks to Mrs Jaqueline Smith (Manager in Histopathology). The Microbiology laboratory for allowing me the time needed to carry out the research, and my colleagues for their support throughout the course of this research special thanks to Mrs Andrea O'Connell (Manager Microbiology laboratory) and Dr Minas (Consultant Microbiologist). Special thanks to Ali Sheikh for his help and support with this research.

I would like to appreciate all my friends and family, special thanks to my parents who set me on this pathway a long time ago; my mother Josephine and my late

father Edward whom I sadly lost last year (Will always love you), I would not have done it without you.

Declaration

I hereby declare that the work presented in this thesis is original work undertaken by me for the Doctor of Philosophy degree, at the School of Allied Health Science, Faculty of Health and Life Sciences, De Montfort University, Leicester, United Kingdom. All other material resources from other works are duly acknowledged.

Edina Chiriseri

Cover image; http://thewellproject.org/sites/default/files/article images/HPV.jpg

Abbreviations and Acronyms

AIDS	-	Autoimmune deficiency syndrome
AJCC	-	American Joint Committee on Cancer
CI	-	Confidence Intervals
CSU	-	Section of Cancer Surveillance
CTL	-	Cytotoxic T lymphocytes
HIV	-	Human Immunodeficiency Virus
HNSCC	-	Head and Neck Squamous Cell Carcinoma
HPV	-	Human Papilloma Virus
HPA	-	Health Protection Agency
HR	-	High Risk
IARC	-	International Agency for Research on Cancer
ISH	-	In situ hybridization
LR	-	Low Risk
NATSAL	-	National Survey of Sexual Attitudes and Lifestyles
NCHS	-	National Centre for Health Statistics
NCIN	-	National Cancer Intelligence Network
NPCR	-	National Program of Cancer Registries
OR	-	Odds Ratios
OSCC	-	Oropharyngeal squamous cell carcinomas
PHE	-	Public Health England
STIs	-	Sexually transmitted infections
SHC	-	Sexual Health Clinic
WHO	-	World Health Organization

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Chapter 1: Introduction

1.1 HPV and oral cancer

Human papilloma infection (HPV) is a known cause of cancer of the cervix. A cervical tumour due to HPV leads numerous nations to introduce HPV vaccine in 2006 in the 9-26-year age groups for both sexes. Recent studies have linked HPV as a cause of cancer especially in the head and neck regions and other regions of the body (McKaig *et al*., 1998). It is still not known how HPV has become a possible causative entity in these regions. A research study has shown an increased number of people engaging in oral sex especially the relatively young adults of 45 and below (Shiboski *et al*., 2005). A possible connection between engaging in oral sex and HPV infection has been suggested, but more research is required in that area (Smith *et al*., 2004).

Head and neck cancer incorporates carcinomas of the mouth, tongue, throat and oropharynx. Most of the research evidence forming the basis of the present study is from data taken from research studies that were carried out in the USA. In the UK, little research to date has examined the role of HPV in cancers of the head and neck. In 2007, head and neck cancers accounted for greater than 45 000 of all new cancers diagnosed in the USA (McKaig *et al*., 1998). Most were diagnosed as oropharyngeal head and neck squamous cell carcinomas (HNSCC), laryngeal and oral cavity carcinomas. McKaig *et al*. (1998), cites that ‘among head and neck sites’, HPV was mainly detected in tumours of the oral cavity (59%), followed by the pharynx (43%), and larynx (33%).

Convincing epidemiological evidence has long established tobacco as a carcinogenic factor in a considerable number of HNSCCs. A tenfold risk for HNSCC was reported for smokers compared to non-smokers by Sturgis and Cinciripini (2007). Moreover, alcohol use increases the risk of HNSCC related with smoking (van Monsjou *et al*., 2005). In addition, heavy alcohol use is an independent entity also linked to increased risk of HNSCC (Ogden, 2005). Historically, most of the HNSCC was attributed to tobacco and alcohol utilization. Tobacco control measures that included clear air laws for indoors and public places have resulted in substantial reduction in cigarette smoking (van Monsjou *et al*., 2005).

The incidence of HNSCC appears to be unaffected by the reduced number of cigarette smokers, due to tobacco control measures in place, especially in young adults. Shiboski *et al*. (2005) showed a marked trend in the increase of young adults with oral tongue cancer and a dramatic rise in oropharyngeal cancer in young adults less than 45 years old in the US. Studies likewise connected HR HPV types to oropharyngeal carcinoma (ORCC) (Mosher *et al*., 2002; Li and Sturgis, 2006). Moreover, studies uncovered that oncogenic HPV DNA was reported in roughly half of oropharyngeal diseases with an especially high number of ORCC in nonsmokers (Sturgis and Cinciripini, 2007). Consistently, more than 90% of HPV-positive oropharyngeal tumours were confirmed to be HPV-16 (Sturgis and Cinciripini, 2007). From literature, the method of transmission of oncogenic HPV to the upper aerodigestive tract is not understood completely, but risk variables, for example, different sexual partners and oral-genital sex were suggested (Li and Sturgis, 2006).

The worldwide vaccination program started in 2006 offering naïve women protection against HPV. Gardasil protects against oncogenic HPV types 6, 11, 16 and 18, while Cervarix a bivalent vaccine protects against HPV types 16 and 18 (Stock, *et al.*, 2013). The suggestions are for utilization of vaccines on both sexes in the 9-26-year olds. Romanowski (2011) assessed the long-term efficacy of the vaccine over 5 years. The study by Romanowski revealed that though antibody titres could wane over time, the clinical protection from vaccination remained high. Notwithstanding, the trial neglected to survey the relationship between vaccinations and other tumours for example, HNSCC.

1.2 History of oral HPV infection

1.2.1 Role of HPV in the Changing Incidence of HNSCC

As indicated by Habbous *et al.* (2013), HNSCC occurrence has increased in recent decades. Data from the US reveal that HPV, which was estimated to account for 16% of HNSCC in the 1980's, now accounts for 60% prevalence; in the most recent study Benson *et al*. (2014) estimated the incidence of HNSCC in the US by 2020 to be higher than that of cervical cancers attributed to HPV. Furthermore, there is a strong correlation between the presence of HPV in cervical metastasis and oropharyngeal HNSCC (Begum *et al*., 2007; Park *et al*., 2010). Though research has reported oropharyngeal cancer to be rising by 60-70%, the prevalence of HPV in other head and neck sites remain lower (Gillison, 2009; Mehanna *et al*., 2012).

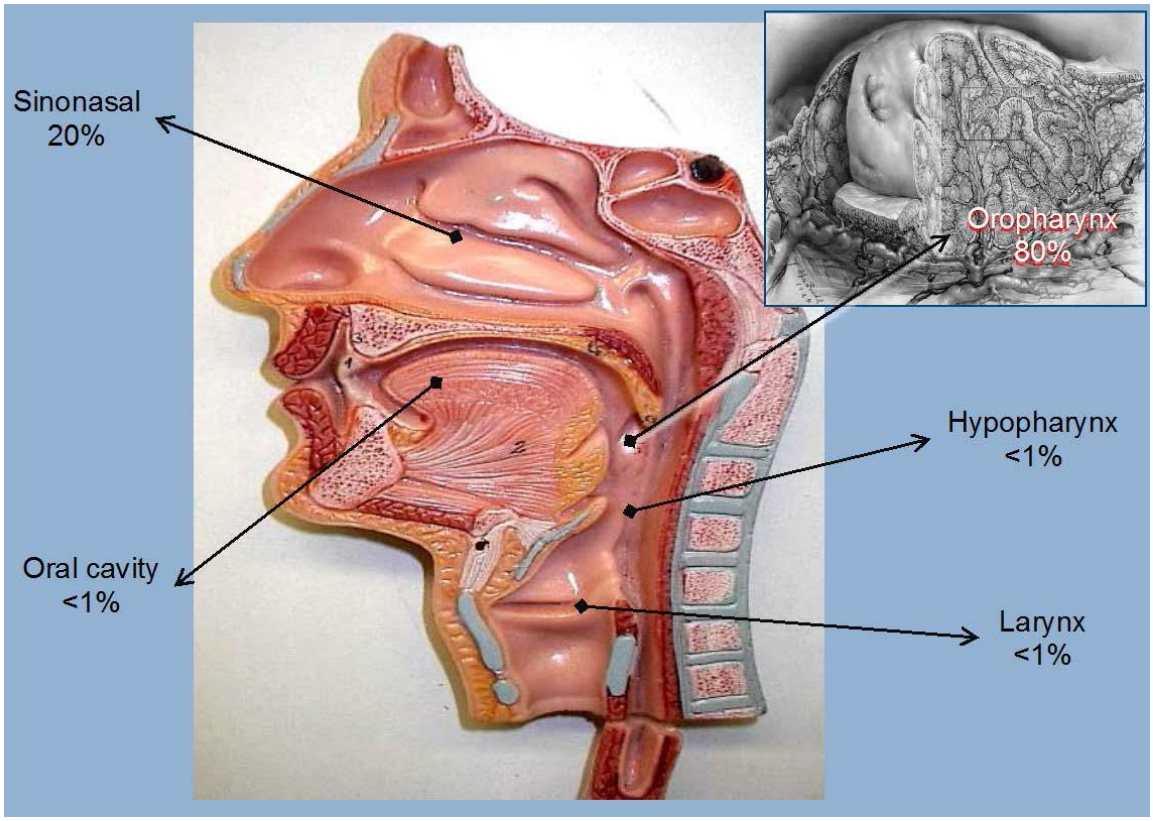

Figure 1: Anatomical distribution of HPV-related HNSCC.

Anatomical distribution of HPV-related HNSCC (brought with authorization from Professor Westra's introduction on May seventeenth (Coughlan *et al*., 2013).

Figure 1 above taken from Westra's introduction highlights the diverse head and neck locations where HNSCC is prevelant (Coughlan *et al*., 2013). The Figure shows oropharyngeal carcinoma to be the most dominant HNSCC with a prevalence of 80%. Though that maybe true, some authors estimate cancer of the oral regions especially mouth and throat to be higher than that of oropharyngeal cancer (McKaig *et al*., 1998).

1.2.2 HPV positive HNSCC and treatment response

HNSCC treatment historically depended on tumour site, tumour nodal status and metastasis and forms the background for the American Joint Committee on Cancer (AJCC) staging system (Habbous *et al*., 2013). Various risk factors are known to modify disease prognosis e.g. smoking and alcohol intake. HPV positive tumours present with late stage AJCC tumours, (Attner *et al*., 2011; Hong *et al*., 2010). Regardless of that, patients positive for HPV respond better to therapy and their overall survival rate is higher in comparison to patients negative for HPV (Lassen *et al*., 2009). A study estimated a 53% overall survival rate for HPV related oropharyngeal carcinomas compared to HPV unrelated carcinomas (O'Rorke *et al*., 2012). Researchers showed a better prognosis for HPV HNSCC, by incorporating various methods such as heterogeneous treatment strategies and variable HPV tumour detection methods (Mellin *et al*., 2000; Schwartz *et al*., 2001).

HPV detection in tumours has gradually evolved over the years and at present clinical testing includes HPV DNA *in situ* hybridization and immunohistochemistry for p16 which is a cell cycle protein upregulated in HPV induced oncogenesis (Snow and Laudadio, 2010). Gillison *et al*. (2000) examined HPV presence in 252 patients diagnosed with HNSCC, which included 60 patients with oropharyngeal cancer. The study showed that HPV was more likely to be detected in the oropharynx. Furthermore, the study revealed that there was 40% reduction in risk of death from all causes when adjusted for age, nodal disease and alcohol consumption. Similar findings were obtained in other retrospective studies (Mellin *et al*., 2000; Schwartz *et al*.,

2001). Moreover, a phase III trial designed to evaluate concurrent cisplatin and standard accelerated-fractionation radiotherapy, evaluated 323 oropharyngeal cancer samples for tumour HPV status (Ang *et al*., 2010). The results showed that cancers positive for HPV (82.4%) exhibited improved overall three-year survival ($p<0.001$) compared to HPV negative tumours (57.1%). Studies elsewhere revealed similar findings, that HPV tumour status was a reliable predictor of better prognosis despite treatment regime (Lassen *et al.* 2011; Posner *et al*., 2011)

Research has suggested that HPV copy number in pre-treatment tumour may have a bearing on the survival rate of patients (Worden *et al*., 2008). In a review containing 42 patients with stage P3 oropharyngeal cancer, high HPV copy number was connected to enhanced response to both induction chemotherapy and chemoradiotherapy (Worden *et al*., 2008). Furthermore, higher HPV viral load was linked to overall better survival for individuals with tonsil carcinoma (Cohen *et al.,* 2008).

1.3 Risk factors for HPV-HNSCC

The history of infection with HPV and the risk factors predisposing individuals to HPV colonisation are still to be understood. According to D'Souza and Dempsey (2011) infection of the oral cavity with HPV in children is uncommon, i.e. < oral HPV% as ≤1% prevalence. Studies have also shown that HPV acquisition increase around sexual debut, with (1.5%, 3.3%) of ages 12 to 15 and ages 16 to 20 respectively positive for HPV in the oral region (D'Souza *et al*., 2009; Smith *et al*., 2007). Besides, carriage of HPV appeared to be

considerably higher in grown-ups with 4.5% of healthy adults testing positive for HPV in their oral regions (Kreimer *et al*., 2010). Therefore, from the sexual debut age (12-15 years) to adulthood there is a steady increase in HPV acquisition which implies that there is a possible connection between engagement in sex and testing positive for oral HPV. In addition, different reviews reported a higher oral HPV predominance for women with cervical HPV infection (Termine *et al*., 2011; Fakhry *et al*., 2006). Individuals with Human Immunodeficiency Infection (HIV), were also reported as having high oral HPV predominance (Kreimer *et al*., 2004). Another study also cites HPV 16 to be present in 1.3% healthy adults; HPV 16 accounted for 85% of all HPV HNSCC cases (Kreimer *et al.,* 2010).

HPV persistence in the oral regions resembles that of anogenital HPV infection and most infections are cleared without intervention (D' Souza *et al*., 2007). It is still unknown how oral HPV is transmitted; how likely diseases are to clear and what components would probably be related to persistence. More studies are necessary to better comprehend oral HPV transmission. According to D'Souza and Dempsey (2011) some dental practices are currently giving oral tumour screening tests. These are carried out by testing saliva for HPV DNA. Data available reveal that the specificity and sensitivity of such tests for pre-cancer analysis are not known. The utility of such test for pre-screening for oral cancer is uncertain as HPV in the oral region does not necessarily result in advancement to malignancy.

Sexual conduct, per D'Souza and Dempsey (2011), has reliably been related with high oral HPV prevalence, supporting the sexual transmission of the infection. A review detailed sexual practices to be co-linear i.e. people with high numbers of sexual partners for one type of sexual act will probably have a higher number of sexual partners for other sexual acts (Allison and Maleck, 2016). D'Souza and Dempsey's (2011) review investigated the association of HPV prevalence with increased numbers of oral sex partners. The results from the study suggested that HPV is most likely to be transmitted by performing oral sex though this is still debatable.

It is still questionable whether HPV is still transmissible to the mouth in other ways. Though there is a strong association between oral sex and HPV it is still not clear whether non-sexual routes exist. According to Smith *et al*. (2007) in children, oral HPV is uncommon even in children born to mothers with vaginal HPV infection. Nonetheless, a review by Rintala *et al.* (2005) discovered oral HPV persistence in mothers to be related with HPV persistence in the oral cavity of their infants suggesting non-sexual HPV transmission route. Furthermore, in a study of college men, open mouth kissing (French kissing) was reported to increase the prevalence of HPV in the oral regions (D'Souza *et al*., 2009). Contrarily, a similar review by Smith *et al*. (2007) revealed conflicting data suggesting further studies to be carried out in that area. Some studies have concluded that oral and cervical infection is very low, i.e. described as the transfer of infection between different sites in the same individual is still very rare (Termine *et al*., 2011; Smith *et al*., 2004).

Despite reviews showing HPV transmission between partners being not yet completed, a review on pregnant woman indicated oral HPV presence in one life partner to be related with a fourfold increase in prevalence of HPV in the other partner (Rintala *et al*., 2006). Moreover, review by Hemminki *et al*. (2000) indicated partners of women with cervical tumour to have a higher rate of tonsillar cancer contrasted to the cervical tumour negative individuals. Besides sexual behaviour being associated with increased HPV carriage, other risk factors have been suggested (Smith *et al*., 2010; Surgis *et al*., 2004). Smoking and drinking habits increase the likelihood of HPV infections to persist. This is due to the perceived tobacco related immunosuppression (Smith *et al*., 2010).

HIV, which likewise cause immunosuppression, is additionally connected with higher HPV oral prevalence (Beachler *et al*., 2013). The rate of HPV infection is different from sexual orientation, oral HPV carriage for men is higher than females (Beachler *et al*., 2013. Klein, (2000) explained the noticed difference to be based on confounding sexual behaviours and hormonal differences between man and women. Furthermore, per D'Souza and Dempsey (2011), oral HPV carriage increases with age which is difficult to comprehend given that HPV prevalence is a sexually transmitted infection. Speculation on this perceived anomaly includes reduced HPV clearance with age and reduced immune system's ability due to age as some of the causes. Table 1 below shows the risk factors associated with HPV related and HPV unrelated HNSCC.

HPV related HNSCC is shown to be on the increase, especially in the tongue and mouth area. Results from the table show HPV related HNSCC to be

connected to more sexual partners, increased engagement in oral sex, and sexual experience at a younger as cited by some authors (Gillison *et al*., 2008; Allison and Maleck, 2015; D'Souza *et al*., 2009). Smoking and alcohol use as shown in Table 1 are not contributing factors to the noticed increase in the number of HPV related HNSCC. Some authors have cited a higher socioeconomic status to be risk factor together with a younger age of 55 and below (Beachler *et al*., 2014). HPV negative HNSCC situated on all head and neck sites is not related with the under 55s', smoking and tobacco use are risks and is related to lower socioeconomic status. Furthermore, results from Table 1 show survival rate to be higher for HPV related HNSCC in comparison to HPV negative HNSCC (Osazuwa-Peters *et al*., 2015).

Table 1: Risk factors for HPV

Factor	HPV related HNSCC	HPV unrelated HNSCC
Incident trend	Increasing	Decreasing
Location	Primarily tonsil and base of tongue/mouth	All head and neck sites
Risk factors	Increased sexual partners Oral sex Early sexual debut	Not linked to sexual activity
Survival	Better	Worse
Smoking history	Not linked	Linked
Alcohol use	Not linked	Linked
Socioeconomic status	Higher	Lower
Median age at diagnosis	Younger age below 55	Older than 60

1.4 Immune system

1.4.1 Immune escape and HPV

The system by which HPV tumours escape the immune system is crucial in establishing persistent HPV infection. Smoking, as will be discussed later (section 1.6), is important in HPV infection and prognosis. In addition, tobacco consumption suppresses immune function allowing persistent HPV infection (Smith *et al*., 2010). Several mechanisms allow HPV to escape the host immune system. According to Stanley (2006) and Stanley (2009), HPV multiplication does not result in instant cell death as protein synthesis and viral gene expression are limited to keratinocytes which are set to die naturally. Pro-inflammatory cytokines, due to the absence of cell lysis, important for migration activation and are not released. In addition, HPV does not have a blood borne phase resulting in reduced numbers of the multiplying virus being presented to the immune system. Therefore, per Stanley (2009) and Kanodia *et al*. (2007), HPV is not perceived by the resistance framework.

In another system, HPV oncoproteins maintain a strategic distance from the impacts of interferon type I known as angiogenic, anti-proliferative, anti-viral, and immune stimulatory (Figure 2). The trafficking of human dendritic cells of Langerhans cells is anti-proliferative and is carried out by the L1 capsid. However, in HPV infection, Langerhans cells that encounter L1 minor capsid are not functionally mature (Fahey *et al*., 2009; Herman *et al*., 2010). Besides, the monocyte differentiation into antigen presenting cells is obstructed by E6 oncoprotein (Figure 2). There is reduction in production of adhesion or soluble

molecules that are important in activation of the movement and operation of Langerhans and dendritic cells (Hubert *et al*., 2005). Decreased expression of proteasome subunits, transporters of antigenic peptides (TAP), major histocompatibility complex class I (MHCI) and low sub-atomic mass proteins (LMP) are examples of HPV's immune evasion (Venuti *et al*., 2011).

Another mechanism of evasion by HPV depicted by Kanodia *et al*. (2007) and Caberg *et al*. (2009), includes balance of chemokines (i.e. MCP-1 expression suppression, IL-8 down regulation, change of CCL20 expression) and pro-inflammatory cytokines expression and hindrance of the Th1 reaction by means of acceptance of a switch from Th1 to Th2 (Figure 2). HPV infection immune failure is suggested to be due to the reason that E7 oncoproteins are similar to various human proteins, e.g. xeroderma pigmentosum group G, complementing protein (XPGC), and the retinoblastoma binding protein (RBP1). Therefore, HPV oncoproteins may not be recognized by the immune system as foreign molecules (Scherly *et al*., 1993).

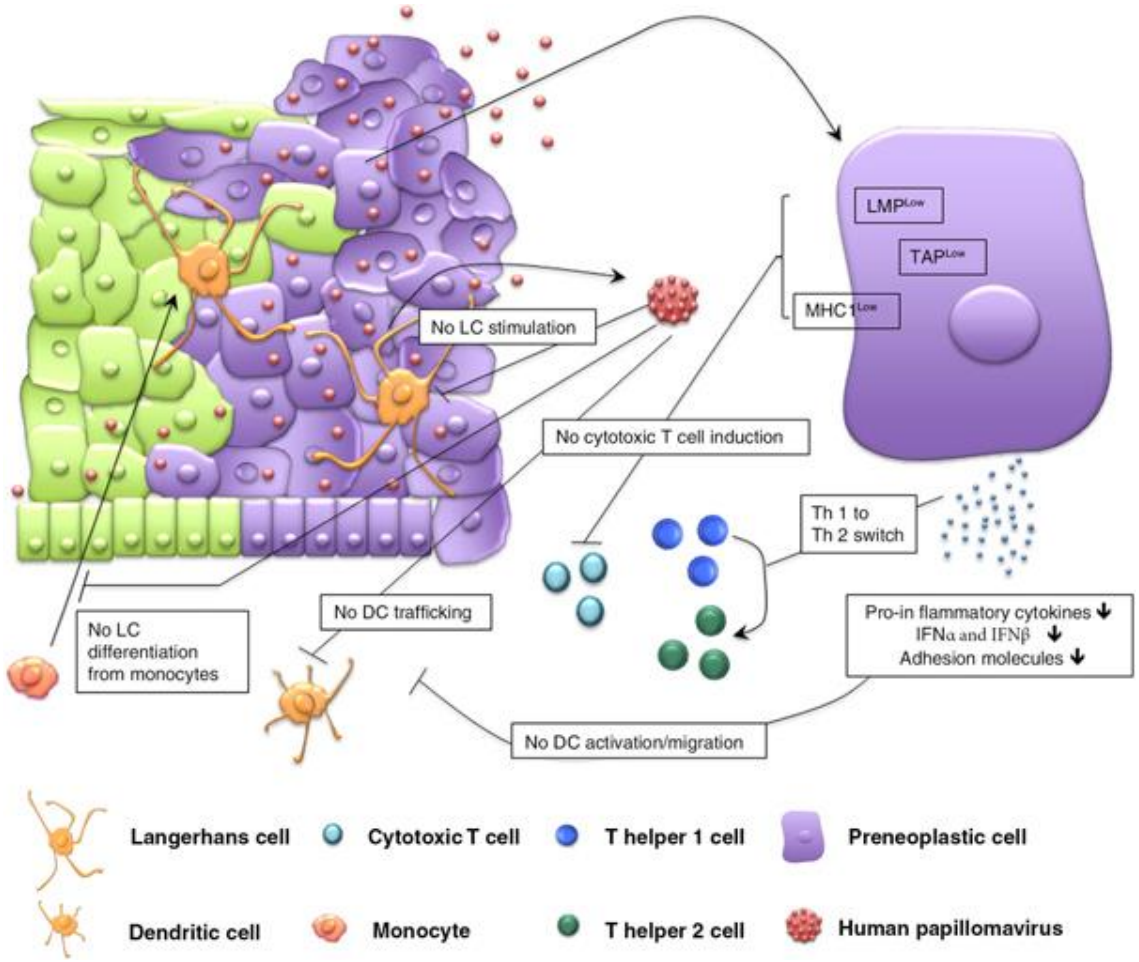

Figure 2: Immunosuppressive mechanisms of escape in the tumour microenvironment. (Fakhry *et al*., 2013)

Several mechanisms were developed by HPV to escape the host immune system. Immune responses though modulation of expression of interferon type I (IFNα, IFNβ), pro-inflammatory cytokines, and adhesion molecules are some of the mechanisms. In the presence of a switch from Th1 to Th2, Th1 response is inhibited. Trafficking and simulation of Langerhans and dendritic cells may be influenced by the capsid of HPV particles. Decreased expression of MHCI, low atomic proteins and transporters of antigenic peptides displayed by cells infected by HPV prompt reduction of T cell induction (Fakhry *et al*., 2013).

1.4.2 Possible impacts of HPV on the immune system of HNSCC patients

Information on the interaction between HPV infection and the host immune system in head and neck cancers is still extremely limited as few studies have been completed regarding that topic. Innate and adaptive immunity's role through the progression and growth of HPV HNSCC is still to be uncovered (Albers *et al*., 2005). Hoffman *et al*. (2006) study data showed that T cell immune reaction against HPV-16 included CD4 and CD8 positive T cells. The reduced recognition of cytotoxic T lymphocytes (CTL) influenced by cell escape results in reduced T cells' capacity to eradicate tumours (Heusinkveld *et al*., 2012). Wansom *et al*. (2010) review demonstrated a higher proportion of CD8 cells and decreased CD4:CD8 ratio in positive HPV 16 cancers. Correlation of CD8 cells between smokers versus non-smokers demonstrated outcomes to be comparative recommending that the distinction in positive CD8 positive cells is associated to HPV not to tobacco usage. Moreover, per that study these outcomes were related with complete tumour response after chemotherapy with enhanced survival. It was concluded therefore that CD8 cells could possibly serve as a factor for prognosis in HPV 16 positive individuals.

As opposed to Wansom *et al*. (2010), later research completed by Heusinkveld *et al*. (2012) utilizing immunohistochemistry to examine different markers (CD4, CD8, CD68 and FoxP3) in tissue specimens of the oropharynx demonstrated no distinction in expression of these markers with respect to HPV status. The outcomes represent that peripheral blood markers are not perfect markers for immune parameters of the tumour microenvironment. HPV and immune status studies examined above, for the most part show interactions between the hosts

immune system and HPV however, the part the of immune reaction leading to development and progression to HNSCC is not known. The studies were also limited to OSCC, that have better survival than other head and neck sites. Peripheral blood experiments carried out by some researchers could not certify results of observations to be directly linked to HPV positive HNSCC rather than other infections present in cancer patients.

According to Vajdic *et al*. (2006) the immune system plays a vital role in viral oncogenesis. Virus related malignancies are more common in people who are immunosuppressed, in this manner the ramifications of safe immune surveillance in the clearance of HPV-positive carcinoma is vital. Efforts are underway to exploit the immune response to treatment-sensitized tumours bearing HPV antigens E6 and E7 using vaccine administration in conjunction with radiation and chemotherapy. The responsiveness of HPV-OSCC to immune surveillance may cause its superior outcome with treatment, and the development of immune-modulating therapies holds promise to further improve an already favourable prognosis.

Current evidence indicates that the improved prognosis of HPV-OSCC is attributable to intact cell cycle machinery that is altered by HPV oncoproteins E6 and E7, which are restored to normal functioning by exposure to chemoradiotherapy. More research is required to establish mechanisms that cause better clinical outcome in HPV-OSCC compared to HPV-negative OSCC. Ultimately, this information will lend itself to targeted, effective and patient-centred treatment with reduced toxicity.

1.5 Smoking and HPV-positive HNSCC patients

HNSCC positive for HPV that is linked to tobacco and alcohol utilization is not the same as HPV-positive HNSCC tumours without these hazard elements, and show better survival. HPV tumour status has been shown to confer a significant prognostic advantage independent of therapeutic approach (Chaturvedi *et al*., 2008). Though patients with HNSCC are regularly treated by surgery, chemotherapy and radiation; research showed that even in absence of chemotherapy or radiation HPV positive tumours are associated with better survival (Licitra *et al*., 2006).

Studies have associated the enhanced treatment response and survival of positive HPV-HNSCC patients with some sociodemographic, clinical and pathologic factors (D'Souza et al., 2010; Byrd *et al*., 2012). These include: male orientation, young age, Caucasian ethnicity, reduced smoking/drinking, oral sex, oropharyngeal area, lymph node metastasis, extended p16 expression, inadequately separated tumour and the inclusion of non-keratinized cells (D'Souza *et al*., 2010; Byrd *et al*., 2012). Furthermore, research by Weinberger *et al*. (2008) showed better survival in HPV positive and p16 positive carcinomas. Byrd *et al*. (2012) undertook a review between 2006 and 2010, on patients with HNSCC in view of that supposition, to distinguish sociodemographic components of HPV-negative patients that relate to clinical elements permitting clinicians to decisively determine patients' HPV status. Several throat doctors predicted a patient's HPV status using clinical and histopathological information of 174 patients, of which 95 were known to be HPV positive. 110 out of 125 patients were effectively distinguished bringing

about a positive estimation of 76-84%. The outcomes likewise indicated male sexual orientation and oropharyngeal site to be altogether connected with the HPV status of the patient. This review affirms the results from D'Souza's past work based mainly on sociodemographic variables (D'Souza *et al*., 2010).

Smoking trends show some significant differences among populations. Diminishing smoking patterns were reported in the Norway, Finland and the Netherlands with Northern European nations indicating a reduction in exposure to conventional risk factors contrasted with Southern Europeans (Giskes *et al*., 2005). HPV HNSCC is currently viewed as a disease mostly common in non-alcoholics and non-smokers. The review by Byrd *et al*. (2012) revealed that 28% HPV related HNSCC patients had a smoking history and 34% were former smokers. Maxwell *et al*. (2010) study supported that finding. Maxwell's study also examined the effect of tobacco use on disease recurrence. The results showed the tobacco smoking group to be at risk of recurrence compared to the non-smoking group. Tobacco smoking and its association with recurrence is quite important in the management of HPV negative HNSCC and is also useful in Asia countries where most HNSCC are driven by smoking. Gillison *et al.* (2012) study likewise showed that p16 positive patients will probably be non-smokers. Besides, these p16 positive patients showed measurably better survival contrasted with p16 negative patients ($p<0.001$). The review showed a 5-year survival of 49% and 19.6% for p16 positive and p16 negative patients independently. In like manner, the probability of progression of disease or demise as a result of the pack-years or total smoking years, was higher even after inclusion of HPV status (Gillison *et al*., 2012).

It is still unknown whether a relationship between tobacco/alcohol utilization and HPV exists. Although increased association have been demonstrated by some studies (Smith *et al*., 2004; Smith *et al*., 2010; Herrero *et al*., 2003) contradicting data was cited in other reviews (Hong *et al*., 2013; Gillison *et al*., 2008) that uncovered no risk of tumour advancement due to excess alcohol and tobacco consumption and HPV status. In the light of these conflicting outcomes, it can be assumed that the potential contribution of alcohol or tobacco use in HPV-related HNSCC is not yet known to date. As already cited (section 1.4), HPV has various mechanisms for host immune system escape and to sustain infection. The use of tobacco suppresses immune function, which allows for infection to persist. Given its immunosuppressive capacities, tobacco use possibly keeps the body from initiating immunologic reactions to eradicate the cancer cells, nicotine, carbon monoxide and hydroquinone induce tobacco inhibitory effects (Arnson *et al*., 2010).

Smoker's reduced response to respiratory tract diseases has been associated with the way that tobacco smoke changes the response of T cells and reduces the phagocytic and executing components of macrophages as well as the survival of these cells (Palmer *et al*., 2005). Reduced cytolytic activity in smokers natural killer cells have been reported (Palmer *et al*., 2005). Likewise, smoking suppresses certain Th1 reactions and encourages Th2 inflammation (Lee *et al*., 2012). For instance, the mononuclear cells of smokers' discharge TNF-α, IL-1, IL-8, and GM-CSF and in addition diminished IL-10 and IFN-γ levels (Lee *et al*., 2012). Furthermore, smoking causes DNA breaks that favour HPV addition at fragile sites potentiating the carcinogenic effect of HPV (Ragin

et al., 2007). These reviews suggest poor outcome due to smoking because of immunosuppressive status that support HPV colonisation and persistence.

1.6 Survival modifiers

1.6.1 Tobacco

The causes of oral cancer have been attributed to several factors. Tobacco, alcohol use and betel quid use are important factors independently or synergistically (Blot *et al*., 1988). Laryngeal tumour is known to be caused by tobacco and alcohol utilization. Smoking causes glottis and vocal cords cancer, while alcohol causes supraglottis cancer (WCRF, 1997). Evaluated numbers ascribed to tobacco smoking for both genders, consolidated results for tumours of the throat, larynx and oral cavity credited to tobacco utilization are from 43 to 60% (Sasco *et al*., 2004). Tobacco consumption of all forms is carcinogenic and research evaluated and confirmed smokeless tobacco to cause oral and pharyngeal carcinoma (Cnattingiuset *et al*., 2005). As per Khlifi and Hamza-Chaffai (2010) the start of the second half of the nineteenth century saw a high increment in tobacco utilization due to large scale manufacturing of cigarettes. In the dawn of the 20th century, cigarettes consumption was low. In the USA, UK and parts of Europe, consumption of cigarettes by men expanded quickly, which was taken years later by women, and finally males in developing countries. At present, cigarette utilization among females in developing nations is still moderately low.

Laws discouraging tobacco utilization publicly decreased the number of smokers in USA and in some European nations significantly (Khlifi and Hamza-

Chaffai, 2010). Smoking is a health burden worldwide, the World Health Organization (WHO) estimates that 1 billion men and 250 million females currently smoke cigarettes, and around 30 million youthful grown-ups begin smoking every year (WHO, 2012). The number of deaths worldwide from tobacco use at present, as indicated by Ajab *et al*. (2008), is approximately 5 million people annually. Based on the assumption that the current smoking patterns remain, smoking will bring about nearly 10 million deaths by the year 2025 with most increases in the Third World, where the yearly mortality from smoking is anticipated to increase to 7 million by the year 2025 (UNDESA, 2004). Tumour due to tobacco smoking is related to the nature of the tobacco or cigarettes utilized (IARC, 1986). Tobacco products contain high levels of tar and nicotine that have been demonstrated to increase larynx carcinoma risk (Khlifi and Hamza-Chaffai, 2010).

Unsafe chemicals contained in tobacco smoke have for some time been connected to infections, for example, nitrosamines, overwhelming metals and polyaromatic hydrocarbons (Pappas *et al*., 2006). Diseases related to tobacco consumption result from repeated exposure to numerous toxic chemicals in cigarette smoke. Adverse pregnancy outcomes, mutagenic, and carcinogenic properties of smoking have been reported (Little *et al*., 2004; Husgavfel-Pursiainen, 2004; Eyre *et al*., 2004). As indicated by Fowles and Dybing (2003) there are five main classes of cancer-causing agents in tobacco smoke. Joined into these 5 are tobacco-specific nitrosamines and polycyclic sweet-noticing hydrocarbons, where solid confirmation for risk to health exist (Hecht, 1999)

Presently tobacco use is an important prognostic factor which is a useful indicator in progression of cancer and survival and exposure to tobacco impact on treatment response and survival. HPV positive patients are classified as low risk and HPV negative individuals with a smoking history are categorised as high risk whilst smokers with HPV-positive tumours are medium risk (Kelly *et al*., 2016). Three-year overall survival is 93% for low risk, 70.8% for intermediate risk, and 46.2% for HR patients (Kelly *et al*., 2016). Finally, HPV positive HNSCC favourable prognosis is affected by smoking history. Concurrently, quantification of tobacco use must be incorporated in future studies of prognosis, treatment and response to therapy.

1.6.2 Alcohol and oral cancer

According to Ogden (2005) alcohol, particularly with tobacco, has been associated with oral cancer for more than half a century. This available evidence has done little to change the drinking habits in Europe and elsewhere worldwide. In 2004, the toll alcohol intake on UK society showed 1.7 billion to be spent on alcohol annually (Prime Minister's Strategy Unit, 2004). Partanen and Simpura (2001) uncovered that an expected 2.9 million people in the UK are alcohol dependent and the quantity of litres of ethanol taken per capita every year inside the United Kingdom has multiplied in the previous 50 years. Although unmistakable exploratory proof is yet missing for pure ethanol to be viewed as a cancer-causing agent, it is a known aetiology for oral carcinoma. According to Ogden (2005), differences in safe drinking levels between different countries makes it difficult when comparing study findings on relationship between alcohol intake and cancer.

The increased number of oral cancer in people that are non-smokers has prompted other sources of alcohol, such as mouth wash. Friedrich and Kristen (2003) study about utilizing pollen tube development tests discovered that numerous mouthwashes were cytotoxic. As mouthwashes are cosmetic not medicinal, manufacturers have no legal obligation to list all ingredients contained. Some mouthwashes have been reported to contain up to 26% alcohol, however, there are difficulties establishing a link due to drinking and smoking habits in some of the participants (Gagari and Kabani, 1995). The rise of mouth cancer coinciding with reduction in smoking has shifted focus back on alcohol (Tarvainen *et al*., 2004). Whilst alcohol intake has remained steadily increasing at the same time as the oral cancer incidences are increasing, the exact mechanism of how alcohol aids in disease acquisition remains unclear.

1.7 The oral mucosa – alcohol mechanisms of action

1.7.1 Mucosal transport; intercellular passage

The permeability of the oral mucosa has been recommended as one reason for oral tumour. Infiltration of cancer-causing agents over the mucosa is increased by alcohol/ethanol. Howie *et al*. (2001) proposed lower alcohol concentration of 15% to be connected with enhancing penetrability contrasted with the higher concentration of 40% due to the latter having a chemical fixative property. (Du *et al*., 2000). Penetrability of tobacco carcinogens is enhanced in concentrations of 25% ethanol (Du *et al*., 2000). However, what is more difficult to explain in the same study is why 50% ethanol decreased the permeability of the floor of the mouth but not the buccal mucosa.

1.7.2 Epithelial cell function

The structure of the oral mucosa is very much described; however, oral epithelial cell function has received less consideration. Epithelial cell response might be critical for understanding oral mucosal disease. Membrane transport can be separated into molecular and micromolecular pathways (Odgen, 2005). The micromolecular pathways can further be subdivided into endocytic and exocytic events. The endocytic events (endocytosis) is split further into phagocytosis (ingestion of molecules larger than 1mm diameter) and pinocytosis (ingestion of molecules below 1mm). The pinocytosis event is receptor or fluid mediated. Axford *et al*. (1999) revealed the evaluation of fluid phase endocytosis inside buccal mucosal cells of alcoholics. The research was undertaken by buccal smear collection from 135 patients comprising of 91 non-alcohol consumers and 44 alcoholics. The cell suspensions were incubated in serum albumin–coated, fluorescent labelled latex microspheres in Hams F10 and culture at 37°C for 60 minutes.

The uptake of microspheres was examined by confocal microscopy, and endocytosed fluorescence levels were determined by flow cytometry. The study revealed endocytosis to be significantly reduced ($p<0.01$) for alcoholics compared to the control subjects. The take-up of microspheres was analysed by confocal microscopy, and endocytosed fluorescence levels were measured by flow cytometry. Restraint from alcohol for a 9-to 14-day demonstrated no change in endocytotic capability. Oral mucosal cells were shown in the study to be fit for endocytosing at 0.02-mm microspheres and that this limit was impacted by alcohol. More studies are required to investigate whether

endocytosis reduction influenced by alcohol is associated with oral cancer regions of the mouth. Besides, it is valuable to evaluate (in a research facility setting or laboratory) the impact of various concentrations of ethanol on endocytic capacity, considering the extensive variety of mixed range of alcoholic drinks consumed by participants in that research. The potentially enormous role of alcohol as a risk factor for mouth carcinoma is not recognised both by the medical experts and the general population. There is data to support that greater awareness is required for both the medical staff and public (Ogden and Graham, 2003).

1.8 Parallels with Cervical Carcinogenesis

According to Petersen (2008) cancer rates and incidence rates are clearly linked to socioeconomical factors with 43% of cancers linked to alcohol consumption, unhealthy diets, inactive lifestyles and infection. Disadvantaged low earning groups of the population are more exposed to avoidable risk factors, such as infectious agents, excessive drinking and smoking. Tobacco consumption, in addition to lung cancer also causes cervical uterine cancer, cancer of the oral cavity, stomach cancer as well as other parts of the body. Tobacco and alcohol consumption synergistically work together to cause cancer of the HNSCC. Yu *et al*. (2015) states that oral and cervical cancers are a global problem disproportionately affecting more men and women in the developing world. Around 528,000 (85%) of these yearly are available in resource poor nations of Africa, Asia and central and south America. Of the 650,000 new tumours analysed yearly of the head and neck 85% are additionally found in resource poor nations. Barriers to cervical and oral cancer

screening in poor countries include lack of health facilities, long waiting times and lack of cost effective follow up options.

Lajer *et al*. (2012) examined 10 samples from HPV positive and 10 HPV negative biopsies from HNSCC patients and compared them to 10 HPV positive and 10 HPV negative biopsies from cervical cancer patients. HPV positive biopsies were first confirmed using PCR. In their research Lajer *et al*. (2012) examined the biopsies using miRNA profiling. Results obtained showed HPV positive HNSCC to have a distinct pattern in comparison with HPV negative HNSCC. HPV positive HNSCC biopsies miRNA profiles were similar to HPV positive cervical cancer biopsies in comparison to HPV negative HNSCC. Moreover, Lajer's study recognized an arrangement of core miRNA profiles the miR-15a/miR-16/, miR-143/miR-145 and the miR-106-363 group which appears to have targets in HPV pathogenesis. This study data substantiates HPV role as an entity both in the oral and cervical regions.

1.8.1 Human Immunodefficient virus (HIV) infection and HPV positive cancer

Oral and cervical HPV prevalence is higher for HIV-positive patients in comparison to negative HIV patients. Beachler *et al.* (2013) collected oral rinse and anal swab samples from over 400 HIV infected patients which showed the prevalence of HPV to be higher in this group compared to the general population. Furthermore, men who had a history of sex with men and women had a higher incidence and persistence of HPV, highlighting the contribution of HPV to the known higher burden of anal compared to oral cancers in this group

of patients. The presence of metastasis to the cervical lymph node is a major determinant for survival for HNSCC patients (Suarez *et al*., 2016). Oral cancer treatment is almost always surgical. Recognizing patients with node positive necks is the most basic question to be answered before surgical resection of the tumour, and for postsurgical treatment and follow up. At present, the best indicator of metastasis is tumour thickness. Two subtypes were discovered that differentiate tumours by risk of metastasis (Bhattacharya *et al*., 2011). These biomarkers will reduce the number of misdiagnosed patients at risk of metastasis in comparison to traditional methods.

1.9 Comparison of the immune microenvironment of the oral cavity and cervix

The decreased prevalence of some sexually transmitted infections e.g. HPV and Chlamydia in the oral region compared to the cervix despite similar frequencies of exposure is still not understood (Fakhry *et al*., 2013). Differences in host immune microenvironments in the two anatomic regions offers a possible explanation. Fakhry *et al*. (2013) carried out research on combined oral and cervical secretions from 39 healthy females. The objective was to compare and correlate the 27 different immune markers in the paired secretion samples. The results showed a high concentration of T-cell related immune markers in the oral region compared to the cervical region. Immune marker profiles significant difference in oral and cervical region offers some explanation on the reduced burden of sexually transmitted diseases in the oral compared to the cervical region.

A mucous layer is a coating of the most part endodermal in origin. It comprises of an epithelium and a basic lamina propria of connective tissue. The layers' line some body areas that are presented to the outside environment and some inward organs. The function of the mucous layers in all regions is for protection, sensation, secretion and thermal regulation**.** The mucous membrane lining the interior of the mouth region termed the oral mucosa comprises of stratified squamous epithelium named oral epithelium and a basic connective tissue named lamina propria. Nanci (2013), depicts the oral cavity as a mirror that gives an impression of an individual's health. Changes in the oral mucosa lining the mouth, occurs in conditions, for example, vitamin insufficiency, diabetes and impacts of unending liquor utilize or smoking, and in addition oral malignancy.

Oral mucosa contains two layers, the surface stratified squamous epithelium and the more significant lamina propria. The epithelium contains keratinized and nonkeratinized oral mucosa; with four layers for the keratinized and the nonkeratinized has two of the more significant four layers with the two superficial layers absent and incorporates a nonspecific superficial layer instead. Nonkeratinized squamous epithelium is prominent on the inner lips, soft palate, inner chicks surface of tongue and floor of the mouth. Keratinized squamous epithelium is confined to the hard palate, gingiva and the dorsal surface of the tongue (Junquiera, 2005).

Keratinization refers to the separation of keratinocytes situated in the stratum granulosum into nonvital surface cells to shape the stratum corneum. Nonkeratinized epithelium contrasted with keratinized contain no superficial

layers demonstrating keratinization. Furthermore, nonkeratinized epithelium may promptly change into a keratinizing type because of frictional or chemical injury, named hyperkeratinisation. Hyperkeratinisation normally affects the buccal mucosa bringing about development of the linea alba, which is an edge of calloused tissue situated at the level where the mandibular and maxillary teeth consolidate. An increased measure of keratin is noted histologically on the surface of the tissue, with orthokeratinised, granular and keratin tissue layers.

Oral malignancies normally emerge as a primary lesion in any of the tissues in the mouth, by metastasis from a distant origin point, or by expansion from neighbouring anatomic structure, for example, the nasal cavity. Moreover, the oral malignancies may begin in any of the tissues of the mouth, and may be of varied histology, for instance, a teratoma, adenocarcinoma, lymphoma from tonsillar or other lymphoid tissue or melanoma from the pigment producing cells of the oral mucosa. Squamous cell carcinomas constitute 90% of all tumours of the oral regions, predominant in tissues that line the mouth and lips. Oral or mouth often includes the tongue however can likewise occur in the mouth areas, for example, the floor of the mouth, gingiva, lips, cheek lining, or palate. Many of the oral carcinomas cannot be distinguished.

Increased numbers of sexual partners and early age at first sexual encounter are among the risk factors for HPV infection. It is essential to note of that around 75 % of oral malignancies have previously been connected to modifiable practices, for example, tobacco and excessive alcohol use. Treatment is in general very effective in early oral cancer diagnosis. In India

chewing betel, paan and Areca is the strongest risk factor for oral cancer; 40% of all malignancies in India are due to oral cancer contrasted with only 4% in the UK (Milgrom *et al*., 2016).

Betel (Piper betle) is a vine leaf in the Piperaceae family which is consumed mainly in Asia, and worldwide by Asian emigrants, as betel quid with Areca nut or tobacco or in paan. Paan is a combination of betel leaf with areca nut with or without tobacco. It is chewed and swallowed or spat out because of its stimulant and psychoactive effects. Individuals chewing betel estimated to be more than five times at risk of oral tumour. The (IARC) reported betel nut as a known human cancer-causing agent. This new finding was from the information gathered and epidemiological examinations from India and Pakistan which unravelled the impact of betel quid with and without tobacco and provided adequate proof of betel as cancer-causing agent.

Oral tumour frequently shows as a non-healing ulcer after 2 weeks especially with HPV type 16 (more than 180 are known), a recognised risk factor for oral carcinoma (Gillison *et al*., 2008). A high number of those with oral tumour does not present with the known stereotypical pattern, oral cancer which was mainly HPV negative cancer was common in those above 50, blacks over whites, males over females, and most cases were from smokers and people who drink alcohol to excess. At present, diagnosis is for mostly those between 30 and 50 years of age, predominantly non-smokers of white race, with more males in comparison to females. A review recently showed that HPV16 is the essential risk factor in newly diagnosed individuals (D'Souza *et al*., 2016). HPV 16 and 18

viruses are known to be responsible for most cervical cancers and worldwide, HPV is a common sexually transmitted infection (Chaturvedi *et al.*, 2011). Oral cancer in this group tends to favour the tonsil and tonsillar pillars, base of the tongue, and the oropharynx. HPV positive cancer compared to that caused by tobacco show a significant survival advantage and disease response to treatment, for example, chemotherapy and radiation (D'Souza *et al*., 2016).

The genital tract for both male and female have mucous membranes. For females, the glans clitoritis and the clitoral hood and the glans penis in males and the inner layer of the foreskin, all contain mucous layers. The urethra for both sexes is also lined with mucous membranes. Mucous membranes are important in absorption and secretion and mucus i.e. fluid produced by the membranes is protective. There are numerous infections, that affect the vagina, including candida diseases, sexually transmitted diseases (STIs) or malignancy. Inflammation and irritation of the vagina, (vaginitis) is due to many vaginal diseases, while involuntary tightening of vaginal muscles during sexual penetration is created by an adapted reflex; HPV, HIV/AIDS, genital herpes and trichomoniasis are examples of STIs that may infect the vagina, and safe sex practices are encouraged by health professionals to counteract STIs (Lloyd *et al.*, 2005).

Cervical carcinoma is preventable and monitored by pap smear screening and HPV immunizations. Vaginal malignancy is exceptionally uncommon and has been linked with old age (Salhan, 2011). The wall of the vagina from the lumen outwards contains firstly a non-keratinized stratified squamous epithelium

mucosa with a covered lamina propria of connective tissue rich in lymphatic channels and veins. A layer of smooth muscle is the next layer, with packs of round fibres inside to longitudinal filaments, and thirdly by an external layer of connective tissue called the adventitia (Arulkumaran *et al*., 2011). The mucosa of the male and female genitalia, as well as the oral regions helps in fighting infection. Similar infections in those regions have been attributed to similarities between the mucosa in those regions. However, Fakhry *et al*. (2013) have investigated the prevalence of T-cell immune targets in those regions and showed increased T-cells in the oral regions compared to the genital regions which explains why despite similar exposure rates to infection, there are high numbers of infections transmitted and reported in the genital area rather than the oral region.

1.10 Treatment and response to therapy

Generally, surgery, chemotherapy and radiotherapy are used to treat HNSCC. HPV related HNSCC has been reported to respond better to treatment and has a better survival rate contrasted to HPV negative HNSCC (Worden *et al*. 2008; Chaturvedi *et al*., 2011). Treatment administrations fundamentally affect and modify the personal satisfaction for HNSCC patients, subsequently an adjustment is required to diminish treatment related morbidity, while utilizing adequate treatment measurements to increase survival (Langendijk *et al*., 2008). According to Benson *et al*. (2014) several deintensification therapy Phase II and Phase III trials are ongoing. In the phase III trial focus is on assessing whether less toxic cetuximab compares to cisplatin when administered with concurrent standard dose radiotherapy. The Phase II trial's

focus on reducing radiation doses and examining dose based response. According to Benson *et al*. (2014) treatment for OSCC patient is not dependent of HPV tumour status. HPV tumour status is only a consideration for participants enrolled in clinical trials. There is no extra treatment for HPV negative OSCC to date regardless of the worse prognosis.

1.11 Epidemiology of HPV infection

According to Joseph and D'Souza (2011) the occurrence of head and neck tumours is increasing universally particularly among men. Head and neck tumours, 90% of which are squamous cell carcinomas have a 2 to 9-overlap increase among men in relation to females (Joseph and D'Souza, 2011). In industrialized nations, HPV HNSCC is increasing rapidly with no less than 80% oropharyngeal growths ascribed to HPV, with 93% more likely than HPV negative carcinomas HNSCC to occur in whites (Joseph and D'Souza, 2011). Also, HPV HNSCC was 16% predominant in non-alcoholic and non-smokers contrasted with 7% in heavy drinkers and smokers, increased in those with no less than 6 lifetime oral sexual partners (46% versus 20%) and those with relatively younger median age at oral cancer diagnosis (54 *vs*. 60 years) (Joseph and D'Souza, 2011).

The natural history of HPV infection for the oral region is not well known to date. Oral cancer incidence varies globally by geographic region and gender; see Figure 5 (Ferlay *et al*., 2015). Oral cancer prevalence rate (per 100,000) in industrialized countries has been reported as higher compared to less developed countries e.g. Australia (40.08), Canada (28.11) and the United

Kingdom (28.09). Significantly high prevalence's of HNSCC in Asian countries e.g. Pakistan 25.98 per 100,000 has been attributed to the practice of smoking and chewing betel. The lowest prevalence is in the African content with some countries having incidence rates as low as 3.20 per 100 000 (Libya and Zimbabwe).

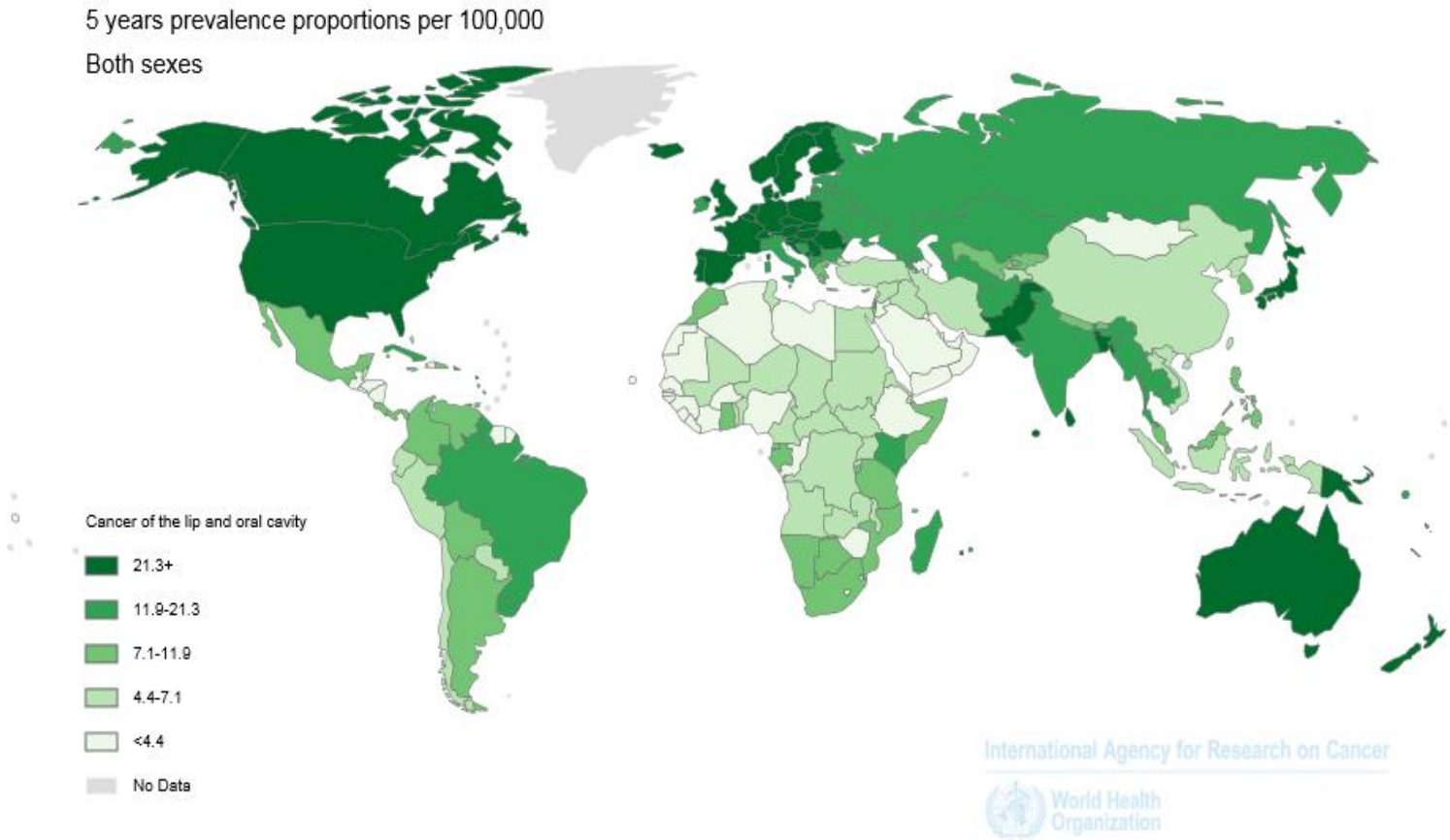

Figure 3: World map from GLOBOCAN portraying age-standardized incidence rates.

(HNSCC incidence rates per 100,000 people) GLOBOCAN 2012: http://globocan.iarc.fr. (03.06.2015).

HPV – known cause of cervical cancer, Increasing HNSCC incidence, change in sexual habits.

Much of the geographical differences in oral cancer prevalence can be explained by tobacco and alcohol intake in the different geographic regions. However, the different anti-tobacco campaigns, especially in the industrial world have seen a huge reduction in tobacco use. This however, is not reflected in the current number of HNSCC incidences which have remained steady or gone up in the past few years. Some authors highlight that at the beginning of the 1980s, there was an increase in tongue malignancies among men younger than 45 years (Hashibe *et al*., 2009; Howlander *et al*., 2009). Similarly, oral HNSCC has been observed in multiple countries in both men and women 50 years or younger (Mork and Glattre, 1998). The increased rates have been observed especially in individuals born after 1950. The tobacco control measures in place remove tobacco as the main carcinogen implicated in these carcinomas, thereby leaving researchers to find out other possible means of transmission. Changes in sexual conduct, resulting in high oral HPV infection, are probably leading to the increased occurrence of HNSCC.

Several HPV subtypes have been shown to cause oral cancer, however types 16 and 18 remain the two main causes of the disease. There is wide variation in estimation of cancers caused by HPV depending on the population and country, study group, year of study, geographic regions, and other environmental factors (Marur *et al*., 2010). A review by Kreimer *et al.* (2005) documented that 26% of HNSCC have HPV detected using PCR, although this prevalence differs by head and neck subunit. HPV as a cause of HNSCC research have provided

both molecular and epidemiological evidence. A study by Joseph and D'Souza, (2011) reported that most oropharyngeal cancers in Europe (14-93%) and United States (36-73%) were caused by HPV. The proportion of HNSCC caused by HPV has been steadily rising over the past 30 years. In a Swedish review, the prevalence of HPV in tonsillar malignancy was cited to have increased from 23% in the 1970s to 68% in 2000 (Hammarstedt *et al.*, 2006). Similar observations were noted in the US where oropharyngeal tissues from 1984-1989 showed 16% HPV prevalence compared with 73% during the 2000-2004 study, demonstrating a 4-fold rise in 20 years (Chaturvedi *et al*., 2011).

HPV plays a major role in causing cancer of the oropharynx, however cancer in other non-oropharyngeal regions cannot be excluded. HPV DNA has been detected in 0 to 31% of laryngeal cancers in the US (Baumann *et al.*, 2009), however higher prevalence has been reported in some European countries (Peralta *et al*., 2010). HPV has been reported in 25% of oral cavity cancers. Some HPV-related oral cavity cancers show clear oropharyngeal involvement, whilst some of these oral cavity cancers are extensions from the base-of tongue.

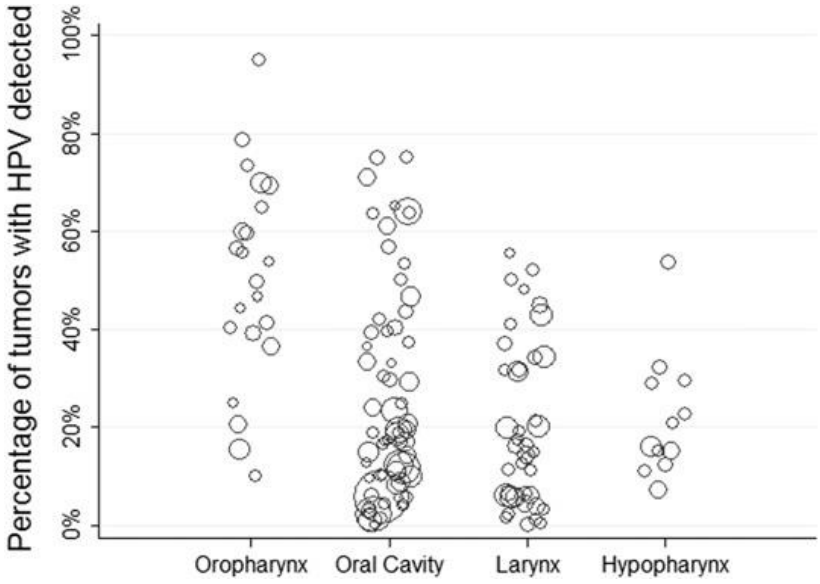

Figure 4: The predominance of HPV in tumours of the oropharynx.
(Picture from Joseph and D'Souza, 2011).

Joseph and D'Souza included more than 25 subjects (oral hole, oropharynx, larynx) and 15 subjects (hypopharynx) and enrolment started after 1990. An individual circle in the image is a representation of the sample size of cases in that study.

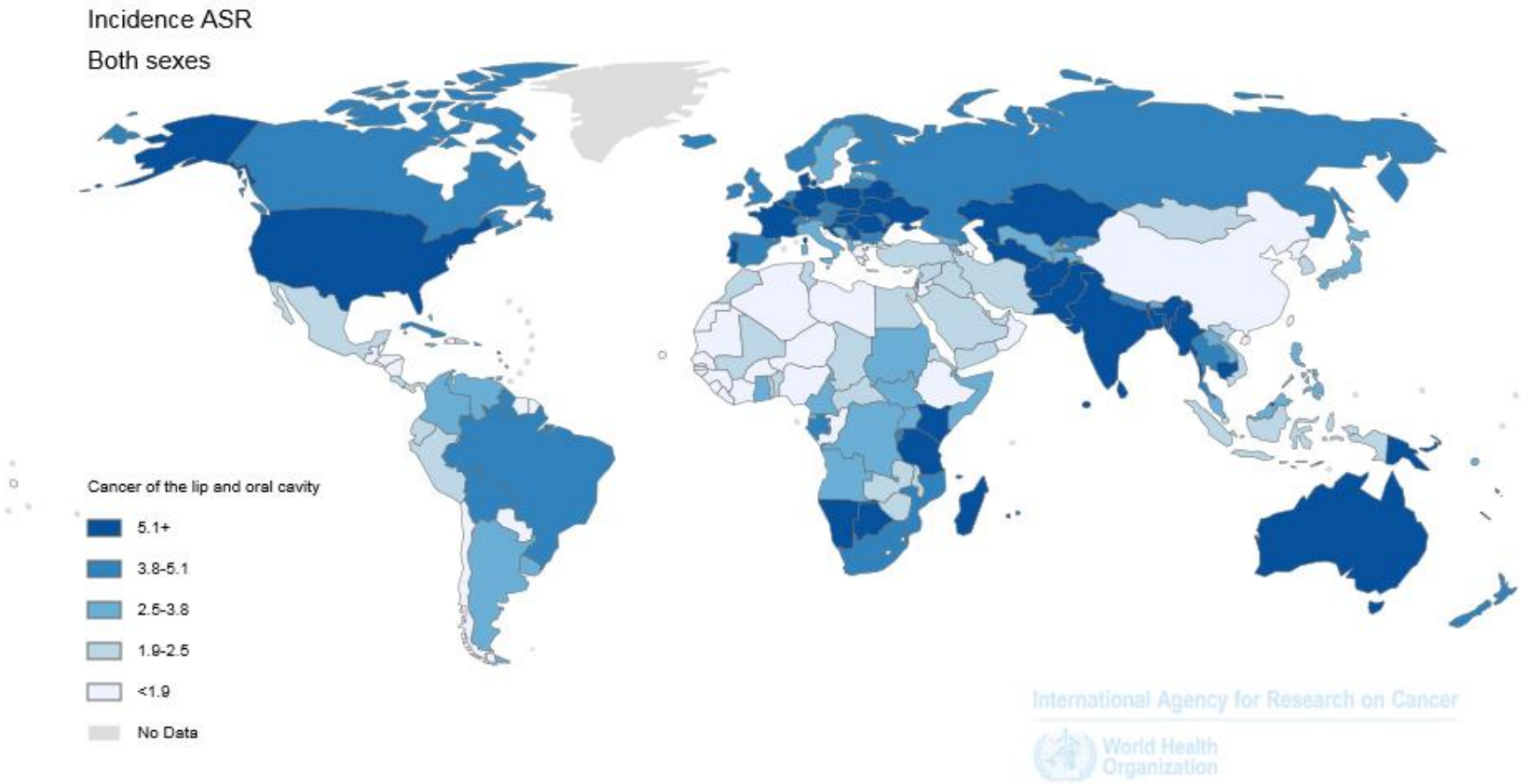

Figure 5: The map of the World from GLOBOCAN depicting age-standardized incidence rates (per 100,000 people).

GLOBOCAN IARC (2012) Estimated cancer incidence; http://globocan.iarc. (2.6.2015)

HPV – known cause of cervical cancer, Increasing HNSCC incidence, change in sexual habits.

There have been no less than 100 types of HPV known to cause illnesses and at least 15 of these have been related to cancer in different regions in the body. The HPV types are separated into high-risk oncogenic HPV types, including HPV-16, 18, 33 with the remaining types categorised as low-risk. The carcinogenicity of HPV in the lower genital tract has been well described, where HPV is the main entity in invasive cervical cancer. Although HPV oropharyngeal cancer has been reported, oropharyngeal carcinoma can emerge without HPV infection and not all oral HPV infections lead to malignant transformation (Gillison and Lowy, 2004). It was evaluated that 4.5% of healthy individuals for both sexes harbour HPV DNA in oral regions (Fakhry *et al*., 2013). Matsushita *et al*. (2011) cited HPV 16 as present in 1.3% of healthy individuals. Oral HPV prevalence is higher among select groups, such as human immunodeficiency virus (HIV) (33%) positive, current smokers (10%) and (7.4%) in people with more than five lifetime sexual partners (Joseph and D'Souza, 2011).

Initial studies suggested oral HPV infections may persist for more than two years. Research by D'Souza *et al*. (2007) investigating oral HPV infections in high risk HIV infected individuals and at risk HIV negative subjects demonstrated HPV infections at baseline as similar to cervical infections and therefore likely to persist for six months. Although more research is required on long term natural history for oral HPV infection, other studies have reported that in comparison to genital HPV infection, most people quickly clear their oral HPV infections (Fakhry *et al*., 2013; Cook *et al*., 2014). Additionally, further

investigations on oral HPV transmission, the average time of clearance, risk factors for persistence are required. The long-term history of oral HPV has not been investigated; starting reviews recommend that, as with genital HPV illness, many infected people may clear HPV oral infections (Rintala *et al*., 2005; D'Souza *et al*., 2011). Additionally, more research on oral HPV transmission, time of transmission, risk factors for HPV persistence are required.

1.12 Risk Factors for HPV positive HNSCC

Epidemiologic studies showed differences in the demographics of patients diagnosed with HPV related HNSCC in comparison with HPV unrelated HNSCC patients (Table 1). As mentioned in (Section 1.11) above patients who have HPV-related cancers are younger, from the higher socioeconomic background, more educated, likely to have more lifetime sexual partners and likely to be of the white race. Moreover, the proportion of HNSCCs that are HPV-related is significantly reduced in black compared with white patients (4% of black HNSCC vs. 34% of white HNSCC) in a recent study (Settle *et al*., 2009). HPV positive HNSCC patients are: likely to have an oropharyngeal primary tumour; to be diagnosed at a late stage; and to have better survival than those with HPV-unrelated HNSCC.

HPV-HNSCC and HPV unrelated incidence rates are 3-fold higher in men compared with women. The historic sex contrast in tobacco and alcohol intake amongst men and women offers a clarification for differences in HPV unrelated HNSCC, however, the higher occurrence of HPV-HNSCC in men is still not

explained. Considering the diverse reasons for HPV-related contrasted with HPV-unrelated HNSCC the risk factors for these diseases are distinctive too. Increased sexual activity (a surrogate for oral HPV introduction) has been related with high chances of acquisition HPV-HNSCC cancer. Because sexual behaviours are collinear (higher numbers of sexual partners for one sexual act often lead to increased numbers of partners for other sexual acts), the odds for HPV-HNSCC have been associated with sexual behaviour, such as increased numbers of lifetime partners for any kind of sex e.g. vaginal and oral sex partners (Joseph and D'Souza, 2011).

Diet has also been implicated as a risk factor for HNSCC. Available research revealed increased intake of fruit and vegetables to reduce the risk of aerodigestive tract associated cancers (Norat *et al*., 2015). Dietary variables represent a huge extent of human cancers, especially overnutrition that lead to weight gain. Since the 1900s information has developed linking diet and cancer. Modern epidemiologic methods and randomized controlled trials have defined the relationship between particular dietary variables and cancer. Obesity, increased consumption of alcohol and red meat, high salt intake has been strongly linked to risk of cancers whilst dietary habits such as high intake of fruits, fibre, calcium, vitamin D, folate and coffee are among factors strongly linked to a decreased cancer risk (Colditz and Dart, 2016).

1.13 The Persistence and Life-Cycle of High-Risk HPV types

The HR HPV types initiate cell proliferation in the basal/ parabasal cell layers and can persist, often for many years, at some sites of infection, however, there is not yet a clear understanding of that observed phenomenon (Zhang *et al*., 2006; Barrow-Laing *et al*., 2010). Though, basal and parabasal proliferation is not required for virus production, it has been suggested increased E6 and E7 levels are relative to the seriousness of cancer (Melsheimer *et al*., 2004). Moreover, the deregulation of genes responsible for the reduction of genetic errors results in integration of viral episomes as observed in many cancers of the cervix (Dall *et al*., 2008; Wilting *et al.,* 2009). Progression of cancer is facilitated when integration preserves E6 and E7 genes, 5th portion on E1 gene and the long control region, yet reduces the capacity of the incorporated genome to express the DNA-restricting protein repressing viral early promoter, and the full-length E1 genome, which can regulate episomal copy number (Van Tine *et al*., 2004).

The end of a life cycle relies upon the epithelial site where infection occurred, and the presence of outside variables, for example, hormones and cytokines (de Jong *et al*., 2004; Gariglio *et al*., 2009). Exploratory models report that infection requires access of viral particles (constituted of viral DNA and two capsid proteins, L1 and L2), which frame icosahedral to the basal lamina, and in addition, the collaboration with heparin sulphate proteoglycans (Culp *et al*., 2006; Johnson *et al*., 2009). According to Schiller *et al*. (2010), epithelial

wounding or micro-wounding is required for HPV infection allowing for the virus to colonise the basal lamina.

Furthermore, a part for the injury healing reaction prompting to the extension of the infected cells was proposed (Ledwaba *et al*., 2004). The dynamic cell division as would happen amid wound repairing is essential for virus entry into the nucleus. The vulnerability for the change zone to malignancy development may be connected to the higher permeability and expansion of the basal cell layers at this metaplastic epithelial site, especially around adolescence and the onset of sexual activity (Grayson *et al.*, 2002). The general speculation has been that lesion formation starts with the infection of undeveloped basal cell, and the life span of the stem cells is a key to persistent lesion formation (Melshermer *et al*., 2004). The hypothesis is plausible for HPV types that are low risk and generally do not cause cancer, however, high risk types can induce basal cell proliferation and it is not clear whether this is necessary.

It has been suggested that HR HPV infection is normal, with over 80% of females infected at some stage in their life, moreover, cervical malignancy rises rarely due to infection. It has been observed that most infections are cleared before deregulated gene expression and secondary genetic errors accumulate (Heusinkveld *et al*., 2012). HPV 16 displays an increased length of persistence contrasted to most high-risk types and thus its increased contribution to cancer risk (Koshiol *et al*., 2006; Schiffman *et al*., 2010). As indicated by Doobar *et al.* (2012), ineffectively comprehended contrasts in cell tropism and disease

progression pattern related with individual HPV types may underlie the higher relationship of HPV 18 with adenocarcinoma (as opposed to squamous cell carcinoma) and its relative infrequency in Cervical Intraepithelial Neoplasia. A review has recommended that the infection of cells in the junctional area between the endo and ectocervix may in certainty underlie the development of numerous cervical cancers (Herfs *et al*., 2012).

HPV 16 has been implicated as a cause of oropharyngeal cancer. Zho *et al.* (2005) reported HPV DNA prevalence in oral rinse of 50% of HPV HNSCC patients. In another review, HPV-positive oropharynx tumour patients were exceptionally likely contrasted with people without malignancy to have HPV oral disease, with high numbers of HPV 16 frequently reported (D'Souza et al., 2009). Moreover, HPV 16 E6 serum antibodies were reported as being common in individuals with HPV positive oropharyngeal cancers in comparison to the general population (Zhang *et al.*, 2016). Zhang *et al.* (2016) also reported HPV 16 E6 seropositivity to be related with people that tested for oral HPV 16 cancer. Most of those participants were of male gender reporting multiple sexual partners.

The epidemiology of HPV related cancers of the cervix was reviewed recently (Mallen-St Clair *et al*., 2016). The results showed similarity between the two types of cancers which were both mainly caused by HPV genotypes 11, 6, 16 and 18. Increased incidence for both types of cancers were associated with risky sexual behaviour with the molecular pathogenesis similar in both cancers.

However, HNSCC appeared to have a longer latency period from the time of infection to presentation of disease compared to cervical cancer. Furthermore, the age of infection was different for HNSCC and cervical cancer, with cervical cancer presenting earlier in life before the age of thirty (Mallen-St Clair *et al*., 2016; Gillison *et al*., 2014).

Another study reported high oral HPV prevalence among HIV infected individuals, with a prevalence of 40% amongst HIV infected individuals compared to 25% for HIV negative individuals (Beachler *et al*., 2012). The few reviews on men who have intercourse with men, demonstrated a fivefold hazard of HPV positive HNSCC in men that engage in sexual relations with men and a fourfold hazard in heterosexual men (Frisch *et al*., 2003; Heck *et al*., 2010). There is reported evidence that performing oral sex on woman increases transmission of oral HPV than performing oral sex on a man (Pickard *et al*., 2012; Beachler *et al*., 2013).

1.14 Prospects for prevention of HPV-driven cancer and indications of HPV vaccination.

HPV type 16 causes more than 90% of HPV-positive OPSCC with other HPV types responsible for a reduced number of HNSCC. In the United States, UK, Europe and other developed regions current HPV immunizations provide immunity against infection with HPV 16, 18, 6 and 11. The HPV vaccine was embraced as the best effective option to counteract cervical malignancy, routinely prescribed for girls 11 and 12 years of age. The development of two

authorized vaccines, quadrivalent (Gardasil) and bivalent (Cervarix) culminated in the production of virus like particles (non-infectious) specific for HPV 16 and 18 aimed at decreasing the incidence rate of cervical carcinoma. These vaccines are targeted against the viral capsid L1, a late protein that is only present toward the start of disease before carcinogenesis. The two vaccines differ in the amount of HPV subtypes against which they target. Both vaccines target HPV 16 and 18, and the quadrivalent counter acting agent also effective against HPV 6 and 11, which additionally cause genital warts and respiratory papilloma.

At present these HPV vaccines are licenced for immunity against cervical cancer and additionally genital warts and anal carcinoma in both sexes. Vaccination against high risk HPV was reported to reduce oral infection and HPV prevalence in those vaccinated by 93.3% (Herrero *et al.*, 2003). Despite immunization effect on HPV related OPSCC not yet being examined, and will be put off in view of decades-long deferral between introduction to HPV and carcinoma recognition; such studies are exorbitant and difficult. Since HPV 16 is the reason for most HPV-positive HNSCC, it is likely that the vaccine would help counteract HPV 16 infection and the resulting HNSCC development when given to people before HPV 16 exposure.

Cervical screening with the Pap smear has identified various women at risk for cervical tumour, and treatment of early infection is lifesaving. The Pap smear is fruitful since the transitional zone of the cervix has a minute transitional zone

range and one swab can test most of the region prone to cervical dysplasia. The anticipated development of cervical dysplasia to cervical carcinoma permits desirable intercession assuming immediate or genuine dysplasia or cervical intraepithelial neoplasia is distinguished, and further Pap smear testing improves Pap specificity. While it is suspected that oropharyngeal carcinoma originates from a dysplastic sore, this is not illustrated, and unfortunately there is no screening examination for the oropharynx like the cervical clinical examination and Pap smear. The tonsil and base of tongue differ histologically from various subsites in the upper aerodigestive tract in having more submucosal lymphoid tissue. In addition, the broad surface area of the tonsil and base of tongue, and the nearness of crypts, prevents inspection of the entire mucosal surface, making testing for dysplasia deficient and of poor predictive value. Screening test for the presence of HPV DNA in an oral/oropharyngeal swab is not sufficient for diagnosis, since most patients will clear the infection and positive results are not an indication of an early silent disease.

The worldwide vaccination program started in 2006 offering women protection against HPV. Gardasil, the quadrivalent vaccine constituting L1 like particles protects against high-risk HPV 6, 11, 16 and 18 was marketed in 2006 and the bivalent vaccine constituting virus like particles offering immunity for HPV 16 and 18 and its marketing started in 2007 (Arbyn and Dillner, 2006). The papillomavirus is made of two viral proteins, real capsid protein or L1 and the

minor capsid protein L2. Virus killing L1 antibodies are focused against epitopes at the surface of the viral capsid and are type specific. Anti L2 antibodies are produced against this segment but they are less potent than the L1 (Frazer and Levin, 2011). As showed by Arbyn and Dillner (2006) the knowledge that the L1 capsid protein could be expressed in eukaryotic cells and accumulate into viral like particles VLPs was a huge stride in immunization progression.

As per Cogliano *et al*. (2005) VLP vaccinations antibody reactions are specific for the HPV type with the minimum response for other HPV types. An estimation in view of 2002 GLOBOCAN estimate of 60000 new cases for every year predicted a 100% immunization success rate against HPV 16 and HPV 18 possibly diminishing HPV rate by 88% yearly (Arbyn and Dillner, 2006). This was based however, on the absence of cross protection as well as the replacement that potentially increases or reduces vaccine efficacy respectively. Follow up trials carried out after five years did not show replacement phenomena, however, given the argument that vaccine protection is effective for 6-8 years more investigations need to be carried out. As indicated by Pons-Salort *et al*. (2013) HPV genotype substitution is unequivocally subject to the suppositions depicting the progression of co-infections.

Firstly, synergistic connections between HPV types influencing the acquisition of infection could be great with HPV genotype substitution, instead of past recommendations. A better understanding of how simultaneous diseases with various types change acquisition time and clearance is fundamental to have the

capacity to foresee the effect of vaccination on genotype distribution. Longitudinal information gathering, especially analysis of infection and co-infection acquisition and clearance is required to understand HPV-immunization effect. Frazer and Levin (2011) revealed that in vaccine trials antibodies decreased by 90% after 2 years but could be boosted by repeat immunisation and high titres.

1.15 Current Controversies in HNSCC Epidemiology

There are several nuances and underappreciated aspects to the epidemiology of HPV HNSCC.

1.15.1 Mischaracterization of HPV positive HNSCC patients

Despite the fact that the normal number of sexual partners is higher among those with HPV-related compared to HPV-unrelated HNSCC, the histories of individual patients sometimes differ enormously, and it is common for patients with HPV-related tumours to have a low number (<6) of lifetime sexual partners. As indicated by Joseph and D'Souza (2011) HPV-HNSCC patients, occasionally have been mislabelled as profoundly sexual or promiscuous. Essential it is important to note that HPV positive individuals are non-smokers and non-alcoholics. In HPV-HNSCC there is evidence suggesting that smoking increases metastasis and disease recurrence therefore counselling for smoking cessation remains a priority for clinicians (Maxwell *et al*., 2010).

1.15.2 Race

Racial disparities in HNSCC incidence and survival remain striking (Settle *et al.,* 2009; Ang, 2010; Schrank *et al.*, 2011). The survival rate for HPV HNSCCs is higher compared to HPV unrelated HNSCC. Because HPV HNSCC is higher in white population compared to black population racial differences in HNSCC survival may be in part or entirely explained by this difference in cause. It is possible that survival of black and white patients with HPV-unrelated HNSCC is similar, however, further investigation of these disparities is needed to understand whether there are differences also in oral HPV acquisition or clearance.

1.15.3 Circumcision

It is still a subject of debate whether circumcision reduces transmission of sexually transmitted diseases. According to Horton and Das (2011), a study on 1,200 heterosexual males showed adult circumcision to significantly reduce the prevalence and incidence of HPV in women. Furthermore, a study showed that circumcised men are significantly less likely to acquire HIV than uncircumcised men (Schoen, 2007). The 2005 randomized trials were terminated after confirmation that there was no longer moral premise to withhold the control group from being circumcised (Schoen, 2007). Furthermore, other studies showed that circumcision prevented sexually transmitted disease e.g. syphilis, Chlamydia and HPV (Schoen, 2007; Fink *et al.,* 2002).

Contrasted with immunization, circumcision can offer numerous protection to various diseases for a lifetime with no requirement for further mediation, for example, booster immunizations. There have been solid claims from groups hostile to circumcision that the prepuce is required for sexual capacity. However, that claim has been questioned by a few reviews that showed no factual contrasts in sexual capacity between circumcised and uncircumcised men (Collins *et al*., 2002; Senkul *et al*., 2004). With respect to HPV, anti-circumcision groups have asserted that though penile area from which HPV can be identified contrasts by circumcision status, HPV is discovered basically on the penile shaft in circumcised man and on the glans penis in uncircumcised men (Fink *et al*., 2002). Therefore, depending on sample collection, HPV risk in circumcised men could be profoundly underestimated (Howe and Storms, 2009).

1.16 Scope of Present Study

1.16.1 Aim

To investigate whether HPV is an entity in HNSCC, to distinguish whether HPV positive HNSCC is impacted by sexual conduct.

1.16.2 Objectives

To establish the prevalence of HPV in biopsies from patients with HNSCC by testing for HPV using PCR and collecting data on age, sex, race, SHC attendance on HNSCC participants.

To collect data on HNSCC incidence and prevalence from public databases such as GLOBOCAN and investigate changing trends in sexual behaviour by collecting data from Health Observatories, East Midlands Public Health Observatory (EMPHO) Information Resource on Sexual Health, NATSAL and other public bodies.

To use the data acquired from testing for HPV and from demographics participant coupled with data from public bodies on sexual trends and HNSCC incidences to investigate the possibility of sexual behaviour being linked to the transmission of HPV into the oral regions. For instance, if the review demonstrated an increased number of the younger population to have HPV related HNSCC and the demographic statistics uncover a high number of young adults practicing oral sex, it could imply that a possible connection exists between oral sex and HPV transmission, which could be the possible explanation for the increase in HNSCC in the oral regions.

Chapter 2 - Methods

2 Study design

2.1.1 HNSCCs patients

The present study, carried out at the Northampton General Hospital NHS Trust in conjunction with the De Montfort University, was carried out on 99 embedded tissue samples from 99 patients diagnosed with squamous cell carcinoma in the mouth and tongue regions; 71 men and 28 women, mean age 55.5 years with age ranges from 23 – 86 years (Figure 6). Most cases were in the 51 to 60-age range (28 for men and 9 for women). All patients were treated at the Northampton NHS Trust between 2006 and 2014, which is a district Hospital in the East Midlands in the UK.

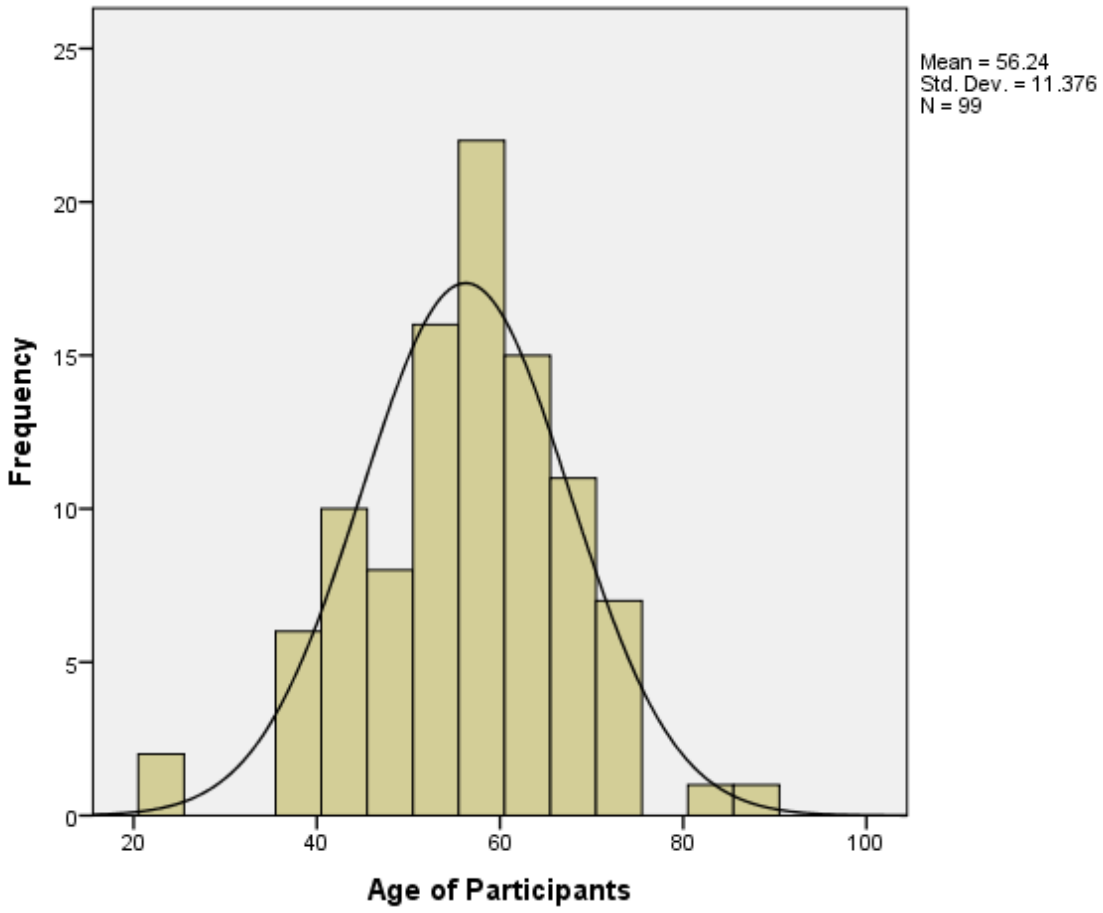

Figure 6: The age distribution of participants.

The mean age of participants for the 99 biopsies tested for the presence of HPV shown in Figure 6 above was 56.2.

The samples, which were obtained from routine surgery for diagnostic or treatment purposes of the patients, were embedded in paraffin wax and stored at room temperature. Clinical data was collected retrospectively from the hospital records. The Ethical Committees of the District Hospital of Northampton, UK Integrated Research Application System (UK IRAS) and De Montfort University approved the study. Statistical data on sexual behaviour and cancer incidences in the UK was also collected from publicly available data

sources; International Agency for Research on Cancer (IARC); GLOBOCAN; National Survey on Sexual Attitudes and Lifestyles (NASTAL); GUMCADv2; Cancer Research UK; Section of Cancer Surveillance (CSU); Public Health England (PHE).

2.1.2 Investigation of tissue blocks for HPV

According to Marur *et al*. (2010) the standard gold method for HPV detection in frozen tissue samples is the E6 oncogene detection using PCR methodology. Retrospective analysis of archival formalin tissue blocks and embedded paraffin tissues can be undertaken using adapted *in-situ* hybridization. In-situ hybridization allows for visualization of HPV directly in tissue specimens. The restriction of the HPV genome to the nuclei is used as a marker to differentiate between the presence of clones in tumour cells from contamination.

PCR, however, does not determine the clinically relevant i.e. differentiation of transcriptionally inactive from active forms. Besides being very sensitive, *in-situ* hybridization is also very specific due to the introduction of various signal amplification pathways with potential to detect down to one viral copy per cell. This study used PCR methodology to determine the presence of HPV in specimens, Micropathology Limited Coventry (Micropathology Ltd). The method used was determined after consideration of the cost implications, accessibility and robustness of the method to produce scientifically acceptable results. The analysis was carried out per QIASymphony (S-392-n); Sigma-Genosys; ABI

3130xl manufacturer's instructions and laboratory standard operating procedures at Micropathology Limited and Northampton Hospital.

2.2 Specimens, data collection and inclusion criteria

The present study included patients of 18 years and above for legal reasons. Participants above the age of 18 of any gender or race who attended the Northampton hospital between the years 2006 to 2014 and diagnosed with HNSCC were included in the research. The study used archived paraffin embedded sections from patients diagnosed with HNSCC. In addition, data was collected from publicly available databases (see section 2.6.1 below) to analyse the relationship between sexual behaviour and carcinoma of the mouth and throat. Inclusion criteria for the present study were that the embedded samples represented the histology of the original tumour and follow-up data were available. All information and data for this research were anonymised.

Blocks for HPV testing were handled by members of staff working in Histopathology department at Northampton hospital; who collected data and assigned identification numbers to specimens prior to analysis. HPV PCR and genotyping were carried out at Micropathology Laboratory Coventry using their standard processing procedure and genotyping methodology briefly outlined below in section 2.4.1 and 2.4.2. Data obtained before and after the research were kept on password locked computers at Northampton Hospital. Data was destroyed as soon as the research was finished.

2.2.1 Sample size

According to the National Institute of Health report good quantitative research endeavours to reduce type I and type II errors (Fox *et al.*, 2009). Type I errors can be described as falsely rejecting a true null hypothesis, i.e. finding an association where there is no association. Type II error is where research data falsely accept the null hypothesis i.e. the opposite of type I error and fails to point out an association which truly is present.

A power search was conducted using a computer based program to calculate the sample size on specimens from 2006 to date that meets the set criteria and 123 samples were identified (see section 2.2). A specimen size calculator with a certainty level of 95% and certainty interval of +/- 5%, with a maximum sample size of 100 were used for the calculation. From the results, the minimum of 80 histology blocks were required to produce statistically significant results. A total of 99 samples were analysed for the present research due to limitation of resources. Testing for HPV and genotyping costed approximately £36 per test therefore, only a maximum of 100 samples blocks could be processed within budget.

2.2.2 Ethical approval and funding

Ethical approval was granted from Northampton General Hospital, De-Montfort University and the Integrated Research Application System UK (IRAS) before the research was commenced. The research was funded by the Microbiology Laboratory at Northampton General NHS Trust.

2.3 Processing embedded tissue samples for referral at Northampton Hospital

Tissue samples for referral were processed per standard laboratory procedure for processing of embedded tissue samples for referral in the Histology department at Northampton Hospital. Embedded samples from HNSCC in paraffin wax were sectioned into 5-10 curls and then inserted into a 1.5ml Eppendorf tube. Only one Eppendorf tube was used per patient. These processed samples were then stored at 4ºC awaiting further analysis by PCR and genotyping (see section 2.3 and 2.4).

2.3.1 Extraction of Paraffin wax embedded fixed tissue and Reagents Used

A total number of 99 samples were processed for HPV PCR and genotyping (Micropathology Ltd). The samples were processed per the standard protocol for PCR and Genotyping in the virology department at Micropathology Ltd. The PCR assay targeted the gene for Major Viral Capsid Protein L1. This method has the potential to pick up most if not all the known HPV genotypes, although the assay is deemed to be more sensitive for some types especially the carcinogenic types compared to the others. Where there is a mixed reaction the assay detects the most prevalent types of HPV where that is designed to have more affinity for. The assay tested for carcinogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 in addition to all other HPV subtypes. The

Extraction protocol, PCR and sequencing methodology used for analysis is outlined below; the detailed methodologies are described in Appendix 1 and 2.

A total number of 99 samples were extracted before PCR and sequencing for HPV. To improve reproducibility samples were extracted automatically using the QIASymphony (S-392-n: QIAGEN, UK) The extraction protocol had five major steps, involving use of Proteinase K, Buffer ATL, PolyA and Isopropanol. Proteinase K, broad-spectrum serine protease with wide specificity is produced by the fungi *Tritirachium album* Limber and is critical in cleavage of the peptide bond nearby the carboxyl group of aliphatic and amino groups. For PCR, Proteinase K reduces contamination and enhances digestion of protein in nucleic acid preparations and the enzyme is also potent in mixtures of chemicals that denature the protein. Destruction of proteins followed by the release of nucleic acids in embedded tissue samples was by use of Proteinase K also important for inactivation of DNases and RNases. For the PCR extraction process Proteinase K was taken from the QiaSymphony extraction kit. Reserve stocks of Proteinase K were reconstituted per the manufacturer's instructions and stored at -70ºC (High Pure Viral Nucleic Acid Kit, ROCHE UK).

Buffer ATL also a tissue lysis buffer is important in purification of nucleic acids. To achieve complete cell lysis an additional step using Buffer ATL combined with proteinase K was incorporated. The preparation of the ATL buffer required gentle warming to dissolve precipitates that form during prolonged storage. Samples extraction on the QIAsymphony included supplementing with phage Φ-

174 and Proteinase K (30µL of a 10-2 dilution per 50mL Buffer ATL). This was the adopted protocol for the extraction of embedded tissue samples for HPV. Poly A was added for extraction for embedded tissue samples. Isopropyl alcohol (IUPAC name 2-propanol), also called isopropanol, is often used in DNA extraction precipitating DNA into a pellet. Isopropanol (20µl) was added to samples, which appeared to be 'bubbly' following the final incubation at 72°C and prior to extraction on the QiaSymphony. Failure to treat such samples with isopropanol would have resulted in incorrect assessment of liquid levels contained in tubes by the carbon-based tips used by the QiaSymphony.

2.3.2 Extraction process of Paraffin wax embedded fixed tissue

Extraction of embedded-tissue samples was carried out by placing 5-10 curls of tissue sections (or scrapings from a block) into a labelled microcentrifuge tube followed by addition of 1ml xylene. The sample was centrifuged for 1 minute at 13,000 rpm. The supernatant was removed carefully without disturbing the tissue pellet and discarded into the designated xylene discard bottle. The tissue was re-suspended in a further 1ml of xylene twice. If the supernatant was still opaque a further xylene wash was carried out. This was followed by addition of 1ml of absolute ethanol to the tissue, centrifugation and removal of the supernatant. The process was repeated carefully by removing as much ethanol supernatant as possible with a pipette tip. Elution Buffer (1ml) was added, centrifuged and the supernatant discarded. The sample was re-suspended thoroughly by vortexing, followed by addition of Buffer ATL (180µl) and

proteinase K (20 µl) then vortexed briefly followed by incubation at 55°C with shaking until the sample appeared homogeneous (3-24 hours).

ATL buffer sometimes required gentle warming to dissolve precipitates that formed during prolonged storage. For longer incubation times, an additional 20 µl Proteinase K was added after the initial 3 hours. If the sample had not fully dissolved after an overnight incubation, a TissueLyser (QIAGEN, UK) with 5mm stainless steel beads was used. The sample was centrifuged briefly followed by addition of 40µL Proteinase K and 200µL of ATL buffer to each sample. This was incubated at 72°C on the heating block for 10min and spun down, then extracted on the QiaSymphony (S-392-n).

2.4 HPV DNA genotyping

2.4.1 PCR and Sequencing Methodology

QIASymphony (S-392-n) (QIAGEN, UK) was used to automatically extract HPV DNA. HPV testing was done with nested oligonucleotide primers routinely sourced from Sigma-Genosys, UK. HPV-genotyping of the amplified PCR products was carried out using the genetic analyser; ABI 3130xl (Applied Biosystems Themofisher Scientific UK). Pellets were re-suspended in molecular grade water giving 50µM concentration of master stock. Primers for HPV_O single target semi-nested PCR were diluted 1:10 from the master stocks to give 5µM working solutions. HPV_N single target primers for semi-nested PCR were diluted per primer degeneracy and they ranged from 0.375µM to 5.75µM. HPV target assays incorporated using a semi-nested PCR. Amplification was

performed on a standard thermocycler, and detection was carried out via gel electrophoresis. If a positive was found *via* gel electrophoresis, then the product was sequenced on an ABI 3130xl genetic analyser. The reagents and primers and equipment used for PCR and sequencing are listed below.

- 5x PCR buffer
- Taq polymerase enzyme (Labmaster)
- 10mM dNTPs solution (Bioline)
- Molecular grade water (Severn Biotech)
- Agarose (Bioline)
- TBE (Invitrogen)
- Ethidium Bromide (Sigma-Aldrich)
- 6x loading buffer (contains 0.25% bromophenol blue/Xylene cyanol, 60% glycerol, 40% water)
- Sure-Clean (Bioline)
- Big Dye version 3 Reaction ready mix (ABI)
- ABI sequencing buffer (ABI)
- Glycogen Solution (Roche)
- EDTA (Sigma-Aldrich)
- NaOAc Solution (Sigma-Aldrich)
- Absolute Ethanol (95% minimum) (Fluker)
- HiDi Formamide (ABI)
- UV Transilluminator

- Standard thermocycler
- Gel electrophoresis
- ABI 3130xl genetic analyser

Mixture preparation (see Table 2 below) was performed in a clean room only and template addition in the general laboratory area or template transfer hoods.

Table 2: Mixture preparation formula for master mix

Reagent	First round quantity (µL)	Second round quantity (µL)
5x Buffer	5	5
dNTPs	5	5
Primer	1	0.5
Water	20	40
Taq polymerase	1	1
PCR aliquot per reaction	30	48

For first round reactions, 20µL of extracted nucleic acid was added to first round mixes. The second-round reactions incorporated addition of 2.4µL of the first reaction mix which was transferred to the respective second reaction in the template transfer cabinet using a filter pipette tip and multi-channel pipette. The internal controls assays run included the appropriate number of positive and negative controls. In-use positive control material for PCR was kept in fridge at 4°C or freezer at -70°C.

The initial stage for PCR included denaturation at 95°C for 2 minutes, followed by a similar process for 30 seconds. The next step was annealing for 30

seconds at 50°C, followed by an extension protocol for 30 seconds at 72°C and procedure repeated for 40 cycles. The second round started with denaturation for 2 minutes at 95°C followed by another denaturation step for 30 seconds at the same temperature. The annealing for the second step was undertaken for 30 seconds at 40°C. The extension protocol for 30 seconds at 72°C was the next step and was repeated for 30 cycles. Gel electrophoresis was carried out by mixing agarose (9g) with 0.5X TBE (300mL) and the solution heated until fully boiling. Ethidium bromide (15µL) was added and once cooled poured and left to set in a tray with a comb. The comb was removed after setting, and then the gel was placed into a gel tank for 23 minutes. Once finished, the gel was checked on a UV transilluminator.

2.4.2 Sequencing

An equivalent volume (40µl) of 'Sure-Clean' reagent was added to the PCR product, vortexed and incubated at 18 to 20°C for 5-10 minutes and centrifuged for 5-10 minutes (13,000 rpm). All tubes were orientated in the same direction with the hinge facing up. The pellet was re-suspended in pre-warmed distilled water, varying the final re-suspension volume in accordance with the PCR reaction yield. Up to 50µl of faint bands were used, 100µl for typical intensity bands and 150-200µl for very bright bands. Molecular biology grade water (4µl), 2µl of purified PCR product (template), 2µl of Big Dye version 3 Reaction ready mix, 1µl of ABI sequencing buffer and 1µl of sequencing primer (5µM) were

added to a labelled 0.2 mL PCR tube by gently pipetting and placed on a thermal cycler.

Parameters for PCR were 30 seconds' denaturation at 96°C, 15 seconds annealing protocol at 50°C, 2 minutes extension at 60°C and the process was repeated for 25 cycles. Stop solution (15µl) and cold (-20°C) absolute (95% acceptable) ethanol (60µl) were added directly to each 10µl sequencing reaction. The solution was mixed by vortexing briefly, and centrifuge at maximum speed (13,000 RPM) for 15 minutes. The supernatant was carefully aspirated, taking care not to disturb the region around the pellet. Cold (-20°C) 70% ethanol 200µl was added and vortex briefly and centrifuged for 10 minutes. The supernatant was aspirated carefully and at this stage as it was vital to remove all the liquid, so it was necessary to perform another pulse spin and then aspirate using a fine tip. The pellet was re-suspended by vigorously vortexing the pellet in HiDi formamide (12.5µl). Finally, a 10µl aliquot of the resuspensions (using an 8-channel pipette) was loaded onto the next available row of an ABI sample loading plate.

2.5 Statistics - Statistical analysis

The test for normality was carried out using Shapiro-Wilk tests and Kolmogorov-Smirnov using age of patients as variable. Kolmogorov-Smirnov test is a nonparametric test useful in analysing equality of continuous, one-dimensional probability distributions and is useful in comparing a sample with a reference probability distribution. Kolmogorov-Smirnov served to analyse the

goodness of fit thereby testing for normality of the distribution, samples were standardized and compared with a standard normal distribution. This was equivalent to setting the mean and variance of the reference distribution equal to the studies sample estimates.

All results for this research were analysed for statistical significance using SPSS for windows. Frequency tables and crosstabulations were used to analyse results using SPSS windows. Multinomial logistic regression was incorporated where appropriate to calculate odds ratios (OR) and 95% confidence intervals (95% CI). For continuous variables, differences in means were analysed using Kruskal–Wallis's test. All statistical tests were considered significant at p-value <0.05 level.

2.5.1 Multinomial logistic regression

Multinomial logistic regression was used to analyse data obtained from PCR analysis of biopsies and patient demographics obtained from 99 participants included in the present study. The technique was used to assess the impact of a set of seven predictor variables on a dependent variable (In this case HPV positivity). The seven predictor variables analysed where smoking, drinking, age, sex, race, gender, SHC attendance and specimen type. Multinomial logistic regression allowed assessment of how well the predictor variables predicted HPV positivity. Multinomial logistic regression was undertaken as the dependent variable (HPV positivity) was not ordered in a particular way i.e. nominal/ categorical and for which there were more than two categories.

Multinomial logistic regression resolves the categorical problem based on the assumption that a linear combination of the noted characteristic and some precise parameters can ascertain the likelihood of each effect of the dependent variable. Information used to generate data for multinomial logit model is case specific i.e. every individual variable has one value for every case. The multinomial logit model is also based on the assumption that it is impossible for the dependent variable to be predicted independently from all independent variables. Therefore, there is no requirement for independent variables to be calculated separately from each other; furthermore, collinearity is assumed, as it is uncommon to separate effect between a few variables. For the model, one category of the dependent variable was chosen as the reference category.

Discrete OR was calculated for all independent variables in each category of the dependent variable excluding the reference category, which is eliminated from the analysis. The exponential beta coefficient indicates the adjustment in OR of the dependent variable being in a reference category, linked with single unit change of the corresponding independent factor. Multinomial logistic regression incorporated a linear predictor function to predict the probability observation outcome. A linear predictor function is a linear function (linear combination) of a set of coefficients and explanatory variables (independent variables), whose value was used to predict the outcome of a dependent variable.

That function usually was included in regression analysis for the present research, in which coefficients were termed regression coefficients. Before the analysis was carried out, the predictor variables were assessed for goodness of fit which is an illustration of the significance of each indicator variable. Multinomial logistic regression was used utilized as there are no assumptions concerning the score distribution for the indicator variables. Outliers influence regression results and to avoid obtaining incorrect predictions data was split into appropriate categories. An example is age where data was subdivided into age categories.

2.6 Investigation of sexual behaviour and carcinoma of the mouth and throat

To investigate whether there is a relationship between sexual behaviour and HPV transmission, publicly available data from sources such as GLOBOCAN, Cancer Research UK and WHO were interrogated for assessment of the relationship between oral sex and HPV transmission (see the subheading 2.6.1 on data sources below).

2.6.1 Data Sources

The data used to assess the various aspects of this research pertaining to sexual behaviour and head and neck cancer were obtained from the (IARC). Data access on this website provides information from databases containing information about cancer recurrence worldwide held by a Section of Cancer Surveillance (CSU) of

IARC. The CSU databases incorporate 3 fundamental databases: GLOBOCAN which supplies access to the latest assessments (for 2012) of the cancer occurrence, mortality and predominance of 28 cancers around the world; CI5 (Cancer Incidence in Five Continents) supplies data on cancer rates recorded by cancer registries worldwide; WHO that presents periodic collection of cancer mortality recorded in chosen nations of the world combined with leading prediction, data and trend analysis provision. NORDCAN presents an up to date long time series of 50 cancers recorded by the Nordic countries, together with facility for advanced prediction and trends analysis. NORDCAN presents recent data of 50 cancers documented by the Nordic countries, in conjunction with leading prediction, data and trend analysis provision.

Finally, SurvCan the final linked project for the IARC presents cancer survival information from cancer registries in low and medium wage regions of the world. Furthermore, UK specific data was sourced from the Cancer Research UK database. This database uses information provided by Cancer registries. The data from cancer registries are crucial for calculating cancer incidence and survival statistics. The Cancer Research UK compiles incidence data produced by the regional cancer registries in England, and the three national registries in Wales, Scotland and Northern Ireland for UK statistics. The Cancer Research UK collects information on incidence complied by the regional registries in UK and Northern Ireland for UK statistics. Due to the complexity of collecting data there is a waiting

period before all data is collected and published.

Mortality, incidence and prevalence data on head and neck cancer was obtained from cancer research UK, GLOBOCAN and Health Observatory. Data for the past five years was acquired from National Centre for Health Statistics, GLOBOCAN, Cancer Research UK and Health Observatory. Information on incidence for periodical patterns (1975-2007), 5-year relative survival rates, and the lifetime likelihood of developing malignancy were acquired from the GLOBOCCAN and Cancer Research UK registries. Northampton County-specific prevalence rates for projecting new cancer cases for the years (2010-2015) and incidence data (1995-2011) were obtained from cancer registries or Public Health England (PHE) and National Program of Cancer Registries as described by the Cancer research UK and National Cancer Intelligence Network. Population data was acquired from GLOBOCAN and the UK Census Bureau, expressed per 100,000 population.

Information on sexually transmitted diseases diagnosed in the genito-urinary medicine (GUM) and on sexual health services was gathered from PHE database. PHE gathers data from (GUM), clinical and community based settings in England. Furthermore, PHE collect information from all local council and NHS hospitals. National Survey of Sexual Attitudes and Lifestyles (NATSAL) performed core national survey on sexual health and attitudes and in-depth qualitative examination of sexual behaviours, community centred measures of sex hormones and sexually transmitted infections (STIs).

NATSAL database was used to obtain information on sexual behaviour in the UK. NASTAL carried out surveys from 1990 onwards in three phases, each phase including at least 12000 adults in the UK and obtained valuable information on sexual experiences, behaviours and views which will help shape our understanding of sexual behaviours in the UK. The NASTAL statistical data is based on interviewing a substantial number of UK adults from 1990. NATSAL 1 carried out in 1990 surveyed 18,876 adults between the ages of 16-59. NATSAL 2 survey was undertaken in 2000 on 12,110 adults between 16 to 44 years of age. Lastly, NATSAL 3 conducted interviews between September 2010 and August 2012 for 15,162 individuals from both sexes aged between 16-74.

The NATSAL methodology for collection of their data is published in 2 books: (Wellings *et al*., 1994; Johnson *et al*., 1994). Some of that data was used for the purposes of this research to assess sexual behaviour and HPV infection. Cases reported by (GUM) to the Health Protection Agency (HPA) provide information of the epidemiology of STIs in the UK. Cases confirmed and diagnosed have been increasing over the last decade. Routine surveillance though necessary, gives partial representation of STI epidemiology. it does not measure the extent of STIs in the population or the number of asymptomatic infections. In addition, it is only limited to socio-demographic and only behavioural information is collected.

To address that issue, urine analysis were carried out from some of NATSAL respondents (5000 for both sexes aged 16-44) and examined for *Chlamydia*

trachomatis, Neisseria gonorrhoea, HIV, *Mycoplasma genitalium* and HPV. The analysis allowed for the measurement of the prevalence of these STIs as well as the identification of demographic, behavioural factors and clinical symptoms associated with each infection. GUMCADv2 is a dataset that gathers STI data from GUM centres and other appointed non-GUM sexual health organisations and that data was utilized for the purposes of this study. Data obtained before and after the research was kept on password locked computers at Northampton Hospital.

2.7 Estimated Prevalence and Incidence of Oral Cancer Cases and Deaths

The exact number of oral cancer cases diagnosed each year worldwide in every nation is unknown because cancer registration is incomplete in some countries. The correct number of oral tumour cases analysed every year worldwide in each country is not known due to incomplete cancer registration in a few nations. Moreover, the current year for which occurrence and mortality information are accessible is three years behind the present year in some cancer registries, for example, GLOBOCAN, because of the time required for information gathering, compilation, and distribution. The most recent data on the GLOBOCAN registry is from year 2012. The methods for obtaining the estimates of incidence, prevalence and mortality rates from GLOBOCAN 2012 was collected following a method on cancer incidence and mortality worldwide

outlined by Ferlay *et al*. (2015). Section 2.7.1 below is a summary on methods of obtaining incidence (GLOBOCAN 2012);

2.7.1 Methods: Incidence (GLOBOCAN 2012)

The methods to estimate the sex and age specific incidence rates of cancer for a specific country fall into one of the following broad categories, in priority order:

- Rates projected to 2012 (38 countries)
- Current rates were adopted to 2012 population (20 countries)
- The weighted mean for the regional estimates (16 countries)
- National mortality estimation was by modelling, using incidence mortality ratios obtained from data recorded in country-specific cancer registries (13 countries)
- National mortality estimation was by modelling, using incidence mortality ratios obtained from data recorded in local cancer registries in neighbouring countries (9 European countries)
- Mortality at national level was determined utilizing modelled survival (32 countries)
- One cancer registry utilized as illustrative of the profile of the nation (11 countries)
- Age and sex rates for all malignancies were isolated by utilizing information on relative frequency of various cancers (by age and sex) (12 countries)

2.7.2 Methods: Prevalence

The 1, 2 and 5 years estimated prevalence for sex and specific cancer for 2012 was compiled electronically by multiplying the comparable estimates for 2008 by estimated incident ratio cases in 2012 adult population to equivalent cases estimated for 2008 (Bray *et al*., 2008). The criteria are defined in Mathers *et al*. (2005).

2.8 Background to National Statistics

National Statistics were initially delivered under the support of the 'Framework for National Statistics' issued in June 2000 later superseded by the arrangements provided in the Statistics and Registration Service Act of 2007. This Act set up the United Kingdom Statistics Authority that is answerable to Parliament and annulled the previous Statistics Commission. The Authority's general aim is to advance and defend the publication of statistical data officially and produce valuable data to the nation and guarantee quality and comprehensiveness of statistics produced. One of the Authority's capacities is to create and keep up a Code of Practice for Statistics and to survey official statistics against this Code before choosing whether they can be classified as 'National Statistics'.

2.8.1 National cancer statistics

The Office for national cancer statistics is UK's national statistical institute and the largest producer for UK’s official statistics. The office independently operates from ministers and communicate to Parliament through the UK

Statistics Authority. The Office for national cancer statistics was formed on April 1st 2008 by the Statistics and Registration Service Act 2007. UK cancer statistics are responsible for activities listed below.

- The data collection, accumulation, examination and dissemination of a scope of key monetary, social and statistic insights about the UK
- The delivery of statistical and methodological guidance for the interest of UK official statistics
- Portrayal of the UK globally as the national statistics foundation

These bodies have released more than 650 diverse statistical data a year that depend on a wide range of techniques and frameworks. Incidence and mortality trends and long-term+- analysis results published on GLOBOCAN 2012 have also been published by Forman *et al.* (2013) and Curado *et al.* (2007).

Chapter 3: HNSCC Results

3.1 HNSCC patients' results

Ninety-nine patients with HNSCC that met the inclusion criteria were identified and these comprised 67 men and 32 women aged between 23 and 86 with a mean age of 55.5 for males and 57.8 for females and an average of 56.2 for both sexes (Figure 6 and 7). Most of the identified cases were carcinomas from the mouth and tongue and all patients were treated at the Northampton general NHS Trust between the years of 2006 and 2014. Carcinomas were prevalent in the 51-60 age groups (28 men and 10 women, see Figures 6, 7). Most of HPV positive carcinomas in the head and neck sites for the present study were identified in individuals below the age of 60.

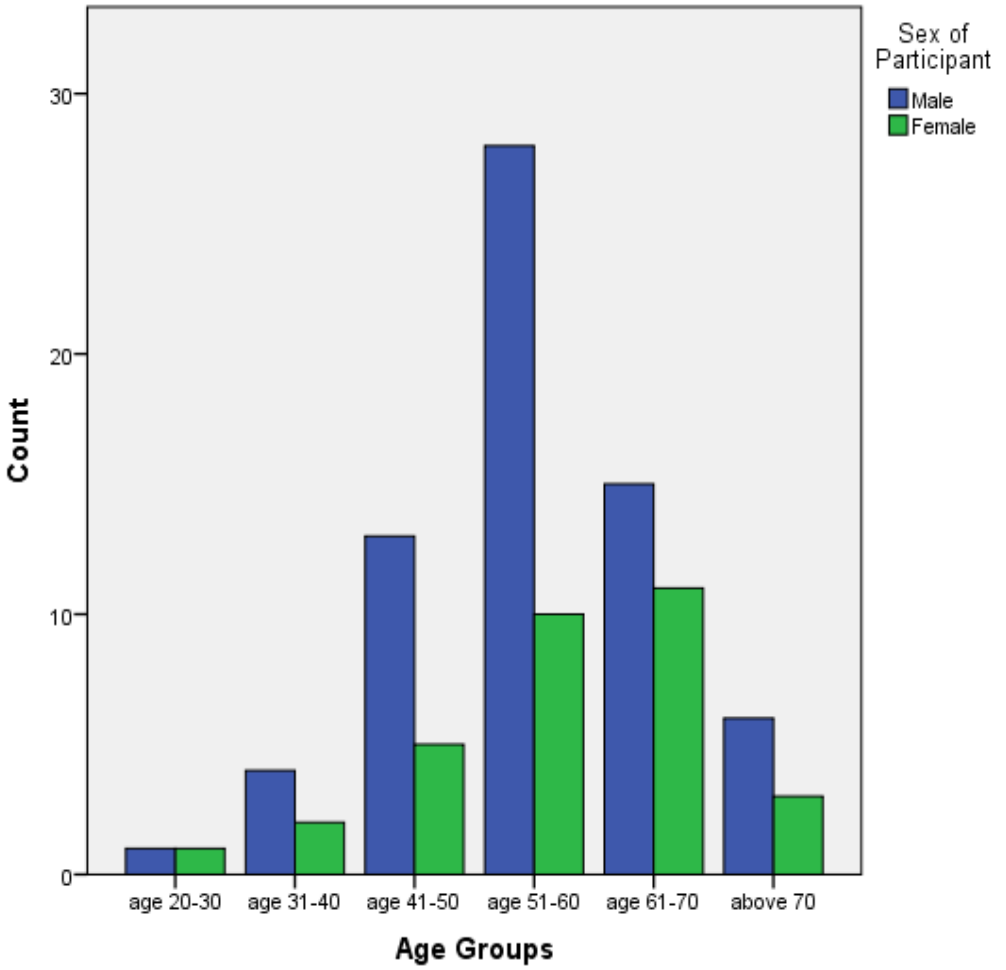

Figure 7: Age groups for males and females included in the research.

Figure 7 above shows the number of participants for males and females. The Figure shows the majority males to be in the 51-60 age groups. It is evident from the graph that most of the participants were in the 51-60 age groups. The average age ranges for both sexes shown in the data above show more female participants to be in 51-60 and 61-70 age groups (10 and 11 participants respectively), whilst most of their male counterparts were in the 51-60 age groups (28). There were more than double the numbers of male participants compared to females (67 and 32 respectively).

Almost all (98%) of the samples were obtained from European whites, with all female participants being white females. For the males, 64/67 (96%) of the participants were white males, two were of Asian and one of black origin. Previous research has shown that most of the HPV positive carcinomas from the head and neck regions are prevalent in white males of higher socio-economic status (Chu *et al*., 2016). The same trend was evident in the present research with 96% of samples being obtained from white males. However, records of the participants' socio-economic status were not available for this research and therefore that aspect could not be verified. A total number of four (5%) of the HNSCC cases had a history of alcohol abuse. The rest (95%) of samples analysed were from non-alcoholics. Table 3 is a summary of patient characteristics for the various age groups. A high proportion (85.9%) of the tested HNSCC cases had no known history of smoking or exposure to tobacco exposure. From the data presented in Table 3 it is evident that most 85(86%) of the biopsies tested were obtained from non-smoking patients. To infer on previous sexual history of patients' records were examined to identify if patients had attended the SHC. The majority of individuals attending the SHC tend to engage in risky sexual behaviour. Risky sexual behaviour can be associated with a history of sexually transmitted diseases and multiple sexual partners (Markle *et al*., 2013). A study by Moreno-Ribera *et al*. (2016) detailed the common characteristics of patients attending the SHC. The majority of patients attending SHCs exhibit high risk sexual behaviours, the most common characteristic being many lifetime sex partners (Kenyona *et al*., 2016). Sexual

behaviours e.g. multiple sexual partners, certain sexual practices, not using safe sex methods and use of illicit substances are high risk behaviours linked with sexually transmitted diseases (Farsi *et al.*, 2015). In addition, Simbayi (2015) noted sexual risk behaviours as early age at first sexual encounter, hazardous sexual practices (i.e., sex without a condom), vaginal, anal, and oral sex, having many sexual partners, taking part in group sex, participating in dry sex or vaginal douching, and having intercourse while bleeding. According to Mercer (2014) SHC patients in general have a history of multiple partners and are prone to engage in more than one type of sexual acts. A total number of 17 patients attended the SHC. All the patients recorded as having attended the SHC were below the age group of 60 with the majority (13) in the 40-60 age groups and the remainder (4) in the 20-40 age groups.

3.1.1 Sexual Health Clinic attendance and HPV positivity

Data shown in Table 3 below represent the number of participants in the different age groups that attended the SHC at some point in the past ten years. The present study examined the total number of participants with previous history of SHC attendance and correlated the results to the presence or absence of HPV (Table 3). These data suggested a possible relationship which was further investigated statistically using multinomial logistic regression. There was a possible relationship between risky sexual behaviour (as measured by SHC attendance and HPV positivity in the biopsies taken from the head and neck oral regions analysed in this research ($p<0.05$) (section 3.7 below).

Table 3: Patient characteristics for the 99 patients tested for HPV grouped according to age groups

	Smoking History		**Alcohol use**		**SHC attendance**		**HPV result**		**Ethnicity**		
	Smoker	None smoker	Alcoholic	Non-alcoholic	Attended SHC	Did not attend SHC	HPV pos	HPV neg	White	Asian	Black
20-40	0	8	0	8	4	4	3	5	8	0	0
40-60	10	46	3	53	13	43	16	40	53	2	0
Above 60	4	31	2	33	0	35	6	29	35	0	0
Total	14	85	5	94	17	82	25	74	96	2	1

The above data in Table 3 shows differences between the younger and older age groups lifestyle characteristics. There were only two smokers below the age of 50, in comparison to twelve smokers above the age of 50. A similar trend was noticed for alcoholics; only one out of the five alcoholics was below the age of 50. All patients attending the SHC were below the age of 60. The majority of HPV positive biopsies were obtained from those below the age of 60, i.e. 19 out of 25 (76%) positive biopsies. Table 4 below shows HPV results for the 99 biopsies analysed. A total of 25 i.e. (25.2%) of biopsies tested were positive for HPV. HPV genotype 16 was the most frequent genotype identified with 23 (92%) of the 25 HPV positive specimens testing positive (see Table 4 below). A total of 19 (28.3%) and 6 (18.7%) HPV positive biopsies were obtained for both males and females respectively.

Table 4: HPV results of the 99 biopsies tested in relation to gender and the HPV genotypes identified.

		Number of Participants		**Total**
		Male	Female	
HPV result	HPV 16 positive	18 (26.8)	5 (15.6)	23 (23.2)
	HPV negative	48 (71.7)	26 (81.3)	74 (74.8)
	HPV 33 positive	0	1 (3.1)	1 (1.0)
	HPV positive unknown genotype	1 (1.5)	0	1 (1.0)
Total		**67**	**32**	**99**

Values in parenthesis indicate percentages.

HPV Genotype 16 was identified in 23 out of 25 positive biopsies tested i.e. 92 percent of all positive HPV specimens

3.2. Detection of HPV-DNA by a PCR-based Method

3.2.1 HPV types and prevalence in archival HNSCC biopsies

In the present study, the 99 cases that met the inclusion criteria comprised: 60 tongue, 12 mouth, 8 vocal cord, 8 laryngeal, 6 oesophagus, 3 pharyngeal and 1 buccal mucosa (Table 5).

Table 5: Total number of biopsies from the specific anatomical oral regions for males and females.

		Sex of Participant		**Total**
		Male	Female	
Specimen type	Tongue	33 (49)	27 (85)	60 (61)
	Mouth	11 (16)	1 (3)	12 (12)
	Larynx	8 (12)	0	8 (8)
	Buccal mucosa	1 (1.5)	0	1 (1)
	Pharynx	2 (3.0)	1 (3)	3 (3)
	Oesophagus	3 (5.0)	3 (9)	6 (6)
	Vocal cord	8 (12)	0	8 (8)
	Tonsil	1 (1.5)	0	1 (1)
Total		**67**	**32**	**99**

Values in parenthesis indicate percentages.

A large number (49%, 85%) of biopsies from the tongue region were obtained from males and females respectively. The second highest number of biopsies tested were from mouth region (16%, 3%) for both males and females respectively.

DNA was extracted from all specimens using the QIASymphony and b-globin sequences were amplified and detected in the PCR assay. Overall, HPV sequences were detected in all 99 specimens in the PCR/reverse blot strip assay. The assay differentiated between positive HPV and negative HPV cases giving a total of 25 positive cases and 74 negative cases. HPV genotyping outlined in Section 2.4 using genetic analyser; ABI 3130xl was successful for 24 out of 25 (96%). One case could not be genotyped by the ABI 3130xl. Genotyping results showed 23 (92%) of the HPV positive samples to be HPV genotype 16 positive. Genotyping results identified one of the biopsies to be HPV genotype 33 positive, however, one of the positive HPV biopsies was not identified to genotype level. This could be due to the assay being designed to target the most common oncogenic genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. There were no multiple HPV infections encountered in this research. The results were consistent with Huang *et al*. (2004) research' which revealed single infections to be associated with high risk oncogenic HPV types such as HPV16 and HPV 33.

Table 6 below shows the different head and neck sites, the total number of biopsies tested for HPV for both male and females and the total number of HPV

positive samples obtained for both males and females. Most of the positive cases were from the tongue (19 cases), followed by the mouth (3 cases). There was only one case each of HPV positive carcinoma identified from the oesophagus, buccal mucosa and pharynx. The table shows that all the positive HPV carcinomas in females (6) were identified from the tongue regions. For positive biopsies, 19 positive biopsies from the present study were identified from males. Of these, most of the HPV positive carcinoma biopsies from males were from the tongue region followed by the mouth region (14 and 3) respectively. All cases that were HPV positive in the pharynx, oesophagus and buccal mucosa were identified from males.

Table 6: HPV positive biopsies distribution in male and female in the different head and neck sites

Sex of Participant			Specimen type			Total
			Tongue	Mouth	Other specimen type	
Male	HPV results	HPV positive	13	3	3	19
		HPV negative	21	7	20	48
	Total		34	10	23	67
Female	HPV results	HPV positive	6	0	0	6
		HPV negative	21	1	4	26
	Total		27	1	4	32
Total	HPV results	HPV positive	19	3	3	25
		HPV negative	42	8	24	74
	Total		61	11	27	99

Table 7 below reveals the number of HPV positive carcinomas in the different age groups for both sexes included in the present research. Overall, most HPV positive cases were those below the age of 60 (18 out of 25 cases). That accounted for 72% of all positive cases. Of the 2 biopsies tested in the 20-30-year age group, one was positive and the other negative. For the 31-40 ages 2 positive biopsies were obtained out of the 4 tested, whilst in the 41-50 age group 5 of the13 biopsies tested in males were also positive for HPV. Furthermore, for the 51-60 age groups 10 of the 38 biopsies were positive for HPV which was the highest number identified in males in comparison to other age groups. Only one female was positive for HPV in that age group. But it is also of interest to note that the highest number of biopsies for males tested fell into 51-60 age range. For the female counterparts in comparison to males, 2 out of the 4 HPV positive biopsies were identified in female patients below the age of 60. Younger age has been linked to HPV positive carcinomas by some authors (Sun *et al*., 2015; Allison and Maleki, 2016; Dahlstrom *et al*., 2015). Allison and Maleki's review showed HPV positive oral squamous cell carcinomas to be common in younger, more educated, white males, and of higher socioeconomic status when compared with individuals suffering from none HPV associated HNSCC. That trend was noted in the present study which revealed most our patients that met our inclusion criteria to be males (71) with most of them below the age of 60. Moreover, all HPV positive HNSCC were identified from white males.

Table 7: The age ranges for HPV positive biopsies (Genotype 16 and 33 combined).

Sex of Participant			**Age Groups**			**Total**
			Age 20-60	Age 60-70	Age above 70	
Male	HPV results	HPV positive	15	3	1	19
		HPV negative	31	11	6	48
	Total		46	14	7	67
Female	HPV results	HPV positive	4	2	0	6
		HPV negative	14	9	3	26
	Total		18	11	3	32
Total	HPV results	HPV positive	19	5	1	25
		HPV negative	45	20	9	74
	Total		64	25	10	99

3.3 Smoking in relation to HPV positive HNSCC

Various research studies have suggested a possible relationship between smoking history and HPV positive results (Schlecht *et al.,* 1999; Falk *et al.,* 1989; Franceschi *et al*., 1999; Blot *et al*., 1988; Marletti *et al*., 1989). The present research investigated the smoking history of participants in relation to HPV positivity. Most participants were non-smokers in 51-60 age groups were also the highest number of HPV positive HNSCC was reported (Figure 8). From the Figure, non-smoking history was strongly associated with HPV 16 positivity ($p<0.001$). Other studies also reported HPV positive HNSCC to prevail in younger age group and with no history of smoking (Gillison *et al*., 2008; Smith *et al*., 2004). Smoking, age and sex results for participants are also tabulated in Appendix 3. The total number of smokers was 11 (11.1%) with the majority 88 (88.9%) being non-smokers. Table 8 shows HPV results between the smokers and non-smokers for both sexes. All females that tested positive for HPV were non-smokers; the majority (89.5%) of males were also non-smokers. Two of the HPV positive males were smokers and whilst the rest (10.5) had no known history of smoking.

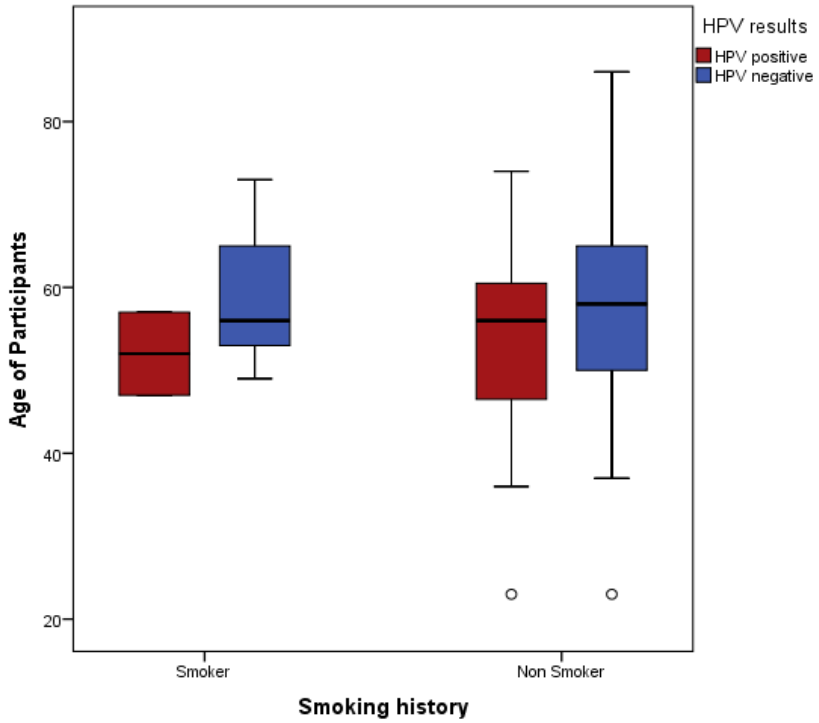

Figure 8: Smoking history and HPV positivity in relationship to age for 99 patients tested for HPV.

Data on smoking history and age of participants was collected from patients' records. The majority of HPV positive participants were non-smokers. The line dividing the box is the median age of participants for HPV positive and HPV negative participants. Bottom and top whiskers of the box plot indicate the highest and lowest age groups for HPV positive and negative patients. The top and bottom of the box indicate the upper and lower age quartiles for HPV positive and HPV negative participants. The Figure shows the median age for HPV positive participants to be lower than that of HPV negative participants with most HPV positive participants being non-smokers.

Table 8: Smoking history and HPV results in relation to sex of participant

Smoking history			**Sex of Participant**		**Total**
			Male	Female	
Smoker	HPV results	HPV positive	2	0	2
		HPV negative	5	4	9
	Total		7	4	11
Non-Smoker	HPV results	HPV positive	17	6	23
		HPV negative	43	22	65
	Total		60	28	88
Total	HPV results	HPV positive	19	6	25
		HPV negative	48	26	74
	Total		67	32	99

3.4 Drinking in relation to HPV results

In the past, HNSCC was generally prevalent in older adults above sixty years of age with a drinking or smoking history. According to Young *et al*. (2015) the face of head and neck cancer have changed dramatically over the past thirty years with a steady decline of tobacco and smoking related HNSCC and steady increase in HPV related HNSCCs. Drinking habits of patients in the present study were examined in relation to HPV positivity. Figure 9 below shows non-alcoholics in the 50-60-year age groups to be mainly associated with HPV genotype 16 positive results. From the Figure, all our HPV 16 positive cases were obtained from individuals with no history of alcohol abuse. None of the alcoholics in the present study were positive for HPV.

The results are consistent with a study by Polesei *et al.* (2011), which reported no clear relationship between drinking status and nasopharyngeal carcinoma risk. In that research, 28 drinks/week of alcohol or more was not significantly associated with nasopharyngeal carcinoma (p=0.06). Moreover, duration of alcohol intake (p=0.74) and age at beginning (p=0.97) were not related to risk of nasopharyngeal carcinoma. Appendix 4 shows all HPV positive participants to have no known history of alcohol abuse, this is in line with Polesei *et al.* (2011) study which showed no relationship between HPV positive HNSCC and alcohol abuse.

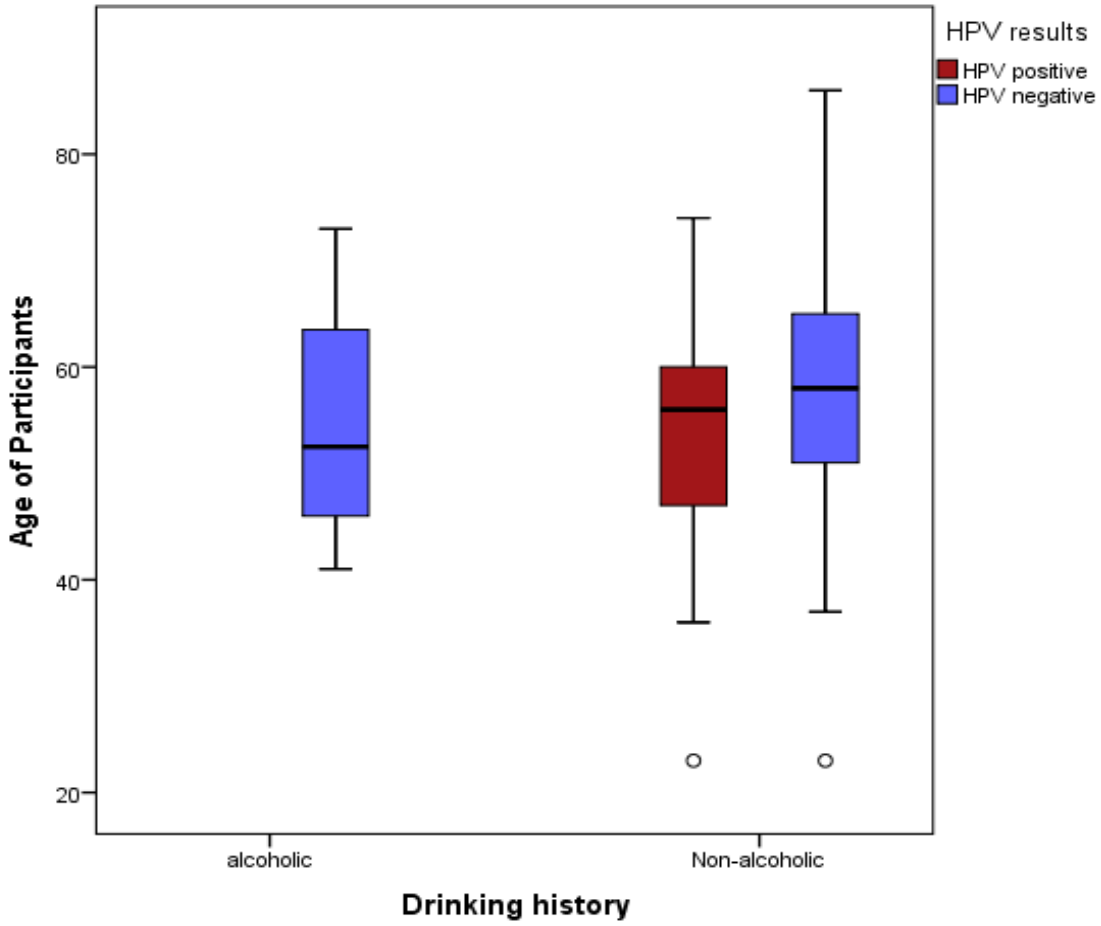

Figure 9: Age of participants, drinking history and HPV positivity.

Figure 9: Age of participants, drinking history and HPV positivity. The line dividing the box is the median age of participants for HPV positive and HPV negative patients. The bottom and top whiskers of the box plot indicate the highest and lowest age groups for HPV positive and negative patients. The top and bottom of the box indicate the upper and lower age quartiles HPV positive and HPV negative patients. The Figure shows the median age of HPV positive participants to be lower than that of HPV negative participants with all HPV positive participants being non-alcoholics. The table showing the actual numbers of participants shown in the Figure is detailed in Appendix 4.

3.5 Racial distribution and HPV results

Research studies have reported racial differences observed with HPV- positive HNSSC (Wang *et al*., 2012; Ragin *et al*., 2011). According to Jiron *et al*. (2012), racial disparities are defined by the National Cancer Institute as adverse contrasts in prevalence, survival, incidence rate, mortality, and tumour burden. HPV positive HNSCC was reported being more prevalent in white males of high socioeconomic status (Jiron *et al*., 2012). Ninety seven percent of participants in the present research were of white origin. In addition, HPV results from the present study showed all the positive HPV biopsies to have been obtained from white males and females, with most participants (76%) being white males (Table 4).

3.6 Mortality and HPV positivity

Data on whether patients survived after diagnosis was sourced from patients' records. Due to the sensitive nature of information required, only the mortality state of patients that demised at the Northampton Hospital was ascertained. Table 9 below shows data on mortality of participants. It is apparent from the Table that much information on patients' mortality was not available for the present study. Only 11 male patients were confirmed to have been deceased with four of the patients, having tested positive for HPV. The other seven patients reported to have deceased were HPV negative male participants. No female HPV HNSCC participants were reported to have deceased. Data had a total of 99 participants, with 13 confirmed to have deceased, 39 confirmed to be

still alive and 47 with no known status of whether they are still alive or not. Because of the unavailability of key data, it was not possible to verify the reports by other research that have cited on reduced mortality rate amongst positive HPV patients compared with their HPV negative counterparts (Cook *et al*., 2011; Ang *et al*., 2010; Chaturvedi *et al*., 2011). It is interesting to highlight that available data of 13 deceased patients reported in the present study had only four participants in the HPV positive category as opposed to nine patients in the HPV negative category. Because of a high number of missing cases, mortality data were not statistically analysed.

Table 9: Sex of Participant, HPV results and Deceased status of participants

Mortality rate			**HPV results**		**Total**
			HPV positive	**HPV negative**	
Deceased	Sex of Participant	Male	4	7	11
		Female	0	2	2
	Total		4	9	13
Not Deceased	Sex of Participant	Male	7	15	22
		Female	5	12	17
	Total		12	27	39
Mortality unknown	Sex of Participant	Male	8	26	34
		Female	1	12	13
	Total		9	38	47
	Sex of Participant	Male	19	48	67
		Female	6	26	32

3.7 Statistical analysis

The Kolmogorov-Smirnov and Shapiro-Wilk tests were carried out using age of the patient as variable to test for normality of the study population distribution. The results obtained were not statistically significant using both Kolmogorov-Smirnov and Shapiro Wilk ($p=0.2$ and $p=0.29$ respectively), indicating normality. Both statistical tests showed the age distribution of the participants to be normally distributed both for males and females together but abnormally distributed for females with a mean age of 56.2 and standard deviation of 11.38. Characteristics of the study population and measurable statistical results are detailed below in Table 10.

Table 10: Statistical investigation and patients' characteristics.

Variables	*n* (%)	Mean (SD)	*p*-Value	HPV pos *n* (%)	HPV Neg *n* (%)	OR HPV pos vs HPV neg
Sex			**p<0.001[m]**			
Male	67 (67.7)	55.2		19 (76)	48 (65)	2.746
Female	32 (32.3)	57.8		6 (25)	26 (35)	
Age		56.2 (12.74)	**p<0.04[m] p=0.408[k]**			
20-60	64 (64.6)			19 (76)	45 (61)	4.605
60-70	25 (25.3)			5 (20)	20 (27)	2.137
Above 70	10 (10.1)			1 (4)	9 (12)	
Race			**p<0.001[m]**			$14.8x10^{6}$
White	96 (97.0)			25 (100)	71 (96)	
Asian	2 (2.0)			0	2 (3)	1.355
Black	1 (1.0)			0	1 (1)	
Smoking			**p<0.001[m]**			0.862
Smoker	11 (11.1)					
Non-smoker	88 (88.9)					
Alcohol			**p<0.001[m]**			
Alcoholic	4 (4.0)			0	4 (5)	$1.3x10^{-7}$
Non-alcoholic	95 (96)			25 (100)	70 (95)	
Sexual health Clinic			**p<0.05[m]**			
Sexual health clinic attendance	17 (17.2)			4 (16)	13 (18)	0.894
Sexual health clinic non-attendance	82 (82.8)			21 (84)	61 (82)	

Values in parenthesis represent percentages

OR- Odds Ratio result using multinomial logistic regression

Association is significant ($p < 0.05$). Bold values represent significant values.

n. Total number of participants

[m]. Multinomial logistic regression

[k]. Kruskal Wallis

Cases differed significantly with respect to sex, race/ethnicity, cigarette smoking and alcohol drinking, SHC attendance, age. For all variables statistically analysed, the assumption was that each variable (age, gender, drinking, smoking, SHC attendance and race) did not influence HPV positivity. See Table 11 for hypothesis test summary for Multinomial logistic regression and Kruskal Wallis statistics test.

Table 11: Hypothesis test summary for all the variables statistically analysed using the Multinomial Logistic Regression.

Multinomial logistic regression-Predicting likelihood of Participant HPV positivity; Null Hypotheses
Males are more likely to be HPV positive compared to females and females.
The prevalence of HPV positives is likely to occur with equal probability across all age groups
The HPV positives are likely to be equally distributed in the different specimen types analysed
Smokers are more likely to be HPV positive compared to non-smokers
Alcoholics are more likely to be HPV positives compared to non-alcoholics
The probabilities of HPV result defined by HPV negative and HPV positive occur with equal probabilities
The prevalence of HPV positives is not likely to be influenced by racial differences.
The prevalence of HPV positives is not likely to be influenced by sexually risky behaviour (sexual health clinic attendance)

3.7.1 Multinomial logistic regression for analysis of the association between patient characteristics and HPV positivity

Multinomial logistic regression was carried out to evaluate the impact of several factors on the likelihood of patients testing positive for HPV. The model contained seven independent variables (age, sex, smoking history, drinking history, race, attending the SHC, specimen type). The full model containing all the predictors was statistically significant ($p<0.004$) demonstrating that the model managed to differentiate between HPV positive and negative participant's likelihood of influence from predictor variables. The model clarified 13.6% (Cox and Snell R squared) and 20.1% (Nagelkerke R squared) and 12.9% (McFadden R Squared) of the fluctuation in HPV status. As shown in Table 10 and Table 12, all the variables uniquely contributed significantly to the model (HPV positive and HPV negative cases).

The predictor coefficient values (B) and odds ratios (OR) for all HPV genotypes combined are shown in Table 12. The second column of Table 12 represents B values obtained in the multinomial regression analysis. These values were used for this research to calculate the probability of a case falling into a specific category. Positive B values represented the positive direction of the relationship i.e. factors that increased the likelihood of an HPV positive result and negative B values represented factors that would decrease the likelihood of an HPV positive result (Pallant, 2013). Therefore, B values below one (unit) represented decreased probability of an HPV positive result and B values above one

represented an increased probability of an HPV positive result. The OR column provided odd ratios for each of the independent variables analysed.

According to Tabachnick and Fidell (2013) OR indicate the odds of being in a selected category of outcome in all cases where the value of the predictor increases by one unit. Categorical predictor variables odds were compared for two categories at a given time. Where more than two categories for each predictor variable was available; comparison with the reference group, usually the group coded with the lowest value was carried out (e.g. for race, white race was coded 1, Asian race was coded 2 and Black race was coded 3). The reference category in the above example is black race (3) and all results were calculated with black race as the redundant group. For the OR, there is another column included in Table 12 (95% confidence interval (CI) for OR. This column represents a 95% confidence interval for OR giving a lower and upper value. The range of values gave an interpretation of the OR the researcher to be confident that the OR encompasses the true value of the OR. The hypothesis for Kruskal Wallis Test was that there was no difference in HPV positivity across all age groups.

3.7.2 Multinomial Logistic Regression results.

Tables 11 and 13 summarise the patient characteristics and Multinomial Logistic Regression Predicting likelihood participant testing HPV positive respectively. The strongest predictor for HPV positivity was age (Table 12) with odds ratio of 4.6 in the 20-60-year age groups compared to those above the

age of 60. This indicated that participants that were of a younger age of below 60 had a 4.6-fold greater chance of being HPV positive compared to those above the age of 60. White race was also another strong predictor for HPV positivity with 6-fold more likely compared to other races OR 28.

The white race has been reported to be a predictor of HPV positivity especially the male gender (Jiron *et al*., 2014). However, the predictions could be due to 97 of the 99 participants being of the white race and reduced data on other races. This was shown by high standard error rate of 4135.0 shown in the table. The results of the model also show alcohol consumption not to be a predictor of HPV positivity in the present study. Alcoholics were 9-fold less likely to be associated with HPV positivity by OR 3.3. This indicated that for alcoholics an individual was over 7- fold less likely to be HPV positive than HPV negative by OR 1.385.

Multinomial regression analysis to assess the difference in HPV positivity between males and females produced a significant result ($p<0.001$). Multinomial regression showed males to be more likely to be positive for HPV, with a coefficient of 1.010 and OR of 2.746 (see Table 12). Regression of the separate HPV genotypes showed males were more likely to be HPV 16 positive, with a coefficient of 0.668 with odds ratio of 1.95. The odds ratios of 2.746 (for all HPV genotypes) and 1.95 (for HPV genotype 16) were both above one, indicating that for every positive HPV 16 result males were 1.95 times more likely to be

HPV 16 and for all HPV genotypes males were 2.75-fold more likely HPV positive compared to females.

Multinomial logistic regression to compare the different age groups showed generally that the younger you are the more likely you were to be HPV 16 positive with HPV negative as reference category (see Table 12 below). HPV positive genotypes were significantly associated with individuals under the age of 60 ($p<0.001$) with a co-efficient value of 1.527 and odds ratio of 4.605. HPV genotype 16 positivity was significantly associated with younger age with a $p<0.05$ for those under forty years of age.

Comparison of HPV positivity for all HPV genotypes and their relationship to smoking showed smoking to be not associated with HPV positivity with a coefficient of -0.144 and odds ratio of 0.862. Therefore, a smoker is 0.862 times less likely to be HPV positive compared to a non-smoker. Comparison of HPV genotype 16 positivity with smoking showed smoking to be less likely associated with HPV 16 positive patients in comparison to HPV negative patients with coefficient value of -2.197 and odds ratio of 0.11. In addition, multinomial regression showed smoking to be likely associated with HPV negative individuals with coefficient value of 2.197 and odds ratio of 9.

Comparison of drinking habits to HPV positive and negative individuals showed alcohol abuse to be mainly associated with HPV negative individuals compared to HPV positive individuals. The coefficient result of 16.5 with an exponential of 150 was obtained for HPV negative individuals drinking habits in comparison to

HPV 16 positive individuals. Furthermore, the results were statistically significant ($p<0.001$). HPV positive individuals were less likely (coefficient -15.7) compared to HPV negative individuals to excessively consume alcohol with likelihood ratio of 6.63 and results significant at ($p<0.001$).

Multinomial regression did not yield significant results ($p>0.05$) for a relationship between specimen type and HPV positivity. Mouth and tongue regions have been cited to have a higher number of HPV positive samples compared to other oral subsites (Montero and Patel, 2015). Researchers have reported a relationship between age and HPV positivity (Patel, 2011; Byers, 1975; Pickard *et al*., 2012; Smith *et al*., 2004). The present study used a Kruskal-Wallis test to confirm the influence of age on HPV positivity. The null hypothesis was that across all age groups there was no difference in HPV positivity. A Kruskal-Wallis Test revealed statistically insignificant result of $p=0.408$ in age and HPV testing positive for HPV. The null hypothesis was rejected confirming the results for the multinomial logistic regression, which showed unequal distribution of HPV positive cases in the younger age groups compared to the older age groups. All statistical calculations were based on the hypothesis' listed in Table 11. The statistical analysis tested all variables with the assumption that each variable (age, race, gender, drinking, smoking, sexual health clinic attendance) did not influence HPV positivity.

Multinomial logistic regression results for those who attended the SHC versus patients that did not attend the SHC showed that there was an increased

likelihood of testing positive for HPV if there was a history of attending the SHC. Therefore, patients not attending the SHC were 0.611 times less likely to be HPV positive (coefficient 0.493) compared to those that attended the sexual health clinic. The result was also statistically significant at $p<0.05$. Table 13 is a hypothesis summary table for multinomial logistic regression results; from the Table, all hypotheses were rejected except for the hypothesis of the probability of males being more likely to be HPV positive compared to females which were confirmed to be true.

Table 12: Multinomial Logistic Regression Predicting participants' likelihood of testing positive for HPV.

HPV results[a]		B	Std. Error	Wald	df	OR	95% Confidence Interval for OR	
							Lower Bound	Upper Bound
HPV positive	Intercept	-21.156	1.361	241.592	1			
	[Male]	1.010	0.585	2.981	1	2.746	0.872	8.647
	[Female]	0[b]	.	.	0	.	.	.
	[Smoker]	-0.149	0.904	0.027	1	0.862	0.147	5.063
	[Non-Smoker]	0[b]	.	.	0	.	.	.
	[Age 20-60]	1.527	1.143	1.784	1	4.605	0.490	43.281
	[Age 60-70]	0.759	1.197	0.403	1	2.137	0.205	22.312
	[Age > 70]	0[b]	.	.	0	.	.	.
	[White race]	17.149	0.000	.	1	$28x10^6$	$28x10^6$	$28x10^6$
	[Asian race]	0.289	4135.041	0.000	1	1.335	0.000	.[c]
	[Black race]	0[b]	.	.	0	.	.	.
	SHC Non Attendance	-0.493	0.725	0.463	1	0.611	0.148	2.527
	SHC Attendance	0[b]	.	.	0	.	.	.
	[Tongue]	1.554	0.723	4.615	1	4.730	1.146	19.525
	[Mouth]	1.203	0.960	1.568	1	3.328	0.507	21.857
	[Other specimens]	0[b]	.	.	0	.	.	.
	[Alcoholic]	-15.793	3223.529	0.000	1	$1.385x10^{-7}$	0.000	.[c]
	[Non-Alcoholic]	0[b]	.	.	0	.	.	.

a. The reference category is: HPV Negative

b. This parameter is set to zero because it's redundant (meaning it is used as a reference category for comparison)

c. Floating point overflow occurred whilst computing this statistic. Its value is therefore set to system missing

B. Coefficients

df. Degrees of Freedom

OR. Odds Ratios

. Results not available – category used as reference category

Std. Error. Standard error of the estimate

SHC. Sexual Health Clinic

Wald.Waldstatistic

Table 13: Hypothesis test summary – Multinomial Logistic Regression.

Hypothesis	Multinomial Regression Result		Hypothesis Accepted/Rejected
	Likelihood	OR	
Males are more likely to be HPV positive compared to females.	Males more likely than females to be HPV positive	2.746	Accepted
The prevalence of HPV positives is equal across all age groups	HPV more likely to be positive in those of younger age groups of 20-60	4.605	Rejected
HPV positives are likely to be equally distributed in the different specimen types analysed	HPV more likely to be identified in tongue and mouth samples	Tongue 4.730 Mouth 3.328	Rejected
Smokers are more likely to be HPV positive compared to non-smokers	Non-smokers are more likely to be HPV positive than smokers	0.862	Rejected
Alcoholics are more likely to be HPV positives compared to non-alcoholics	Alcoholics are less likely to be HPV positive compared to non-alcoholics	$1.385x10^{7}$	Rejected
HPV HNSCC is not likely to be influenced by racial differences.	White race is more likely to be HPV positive	$28x10^{6}$	Rejected
HPV positivity is not influenced by sexually risky behaviour (SHC attendance)	Attendance of SHC is more likely associated with HPV positivity	0.61	Rejected

Chapter 4: Oral Cancer Incidences

Mortality data from 1930 to 2011 for the UK and worldwide were obtained from the IARC, GLOBOCAN and Cancer Research UK databases. Public Health Observatories England, PHE and GLOBOCAN were searched for data on long-term incidence from (1975-2011) and for relative survival rates for 5-years. Northampton county specific data was obtained from cancer registries, the Oxford Cancer Intelligence Unit and PHE profile 2015. NASTAL surveys, NATSAL 1, 2 and 3 surveys provided data on sexual trends in the UK recorded on the NATSAL website. The UK Census Bureau provided population data in the UK. All incidence rates are age standardized and expressed per 100,000 population.

4.1 Trends in Oral Cancer incidences

The information analysed demonstrated that there was a general increase in the number of oral malignancy cases diagnosed yearly worldwide. The noted increase was observed in the HNSCC for both men and women. The expected number of new oral cancer cases worldwide are shown in Figure 10 below. The Figure shows Europe and Asia to have the highest number of oral cancer cases for both sexes, with Asia having an estimated 64.6% and Europe having an estimated 16.2 of the cases. The lowest estimated oral cancer incidences were in Africa and the Oceania with 5.9% and 0.6% respectively. Table 14 below shows the overall number of cancer incidences to be on the increase in the next

few years in both sexes, with a GLOBOCAN estimate of 18,499 in 2012 set to increase to 19,393 in 2015.

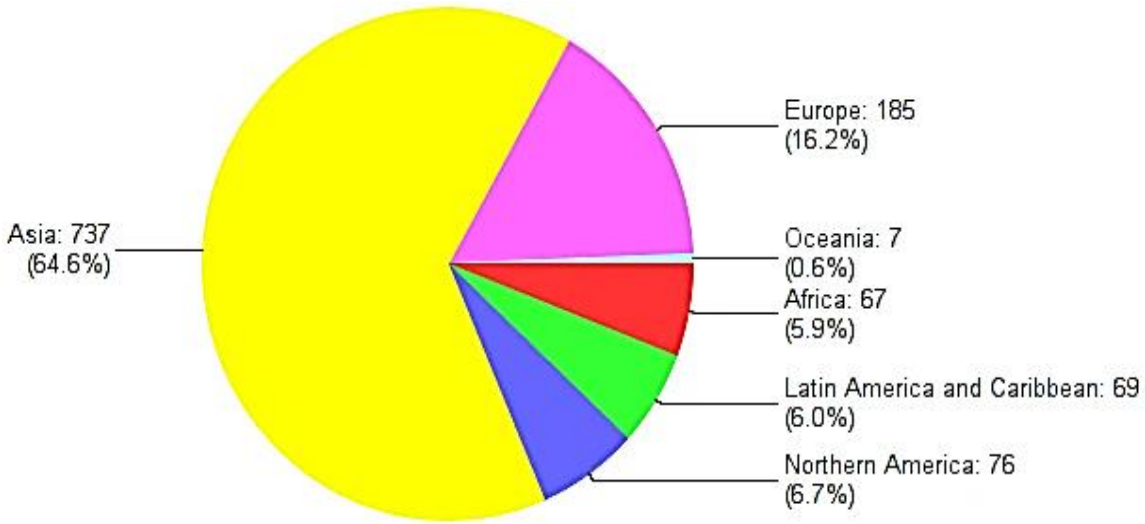

Figure 10: Global Oral cancer estimates.

Global Oral cancer estimates (x1000) for both sexes and all ages; nasopharynx, other pharynx, lip, oral cavity, larynx, oesophagus.

GLOBOCAN 2012 (IARC) (3.6.2015)

http://globocan.iarc.fr/data/GLOBOCAN_PCS_67879510.png

Table 14: Estimated number of new cancer cases worldwide for males and females (GLOBOCAN).

Year	Estimated number of new cancers (all ages)	Male	Female	Both sexes
2012		12783	5716	18499
	ages < 65	5510	1957	7467
	ages >= 65	7273	3759	11032
2015		13458	5935	19393
	ages < 65	5563	1978	7541
	ages >= 65	7895	3957	11852
	Demographic change (2012 to 2015)	675	219	894
	ages < 65	53	21	74
	ages >= 65	622	198	820

GLOBOCAN 2012 (IARC) (3.6.2015). Population forecasts were obtained from the United Nations, 2012 revision, World Population prospects. Numbers were compiled using age-specific rates for corresponding populations for 10 age-groups

The WHO database was searched to examine trends in oral cancer incidences in selected European countries in comparison to the UK for both sexes.

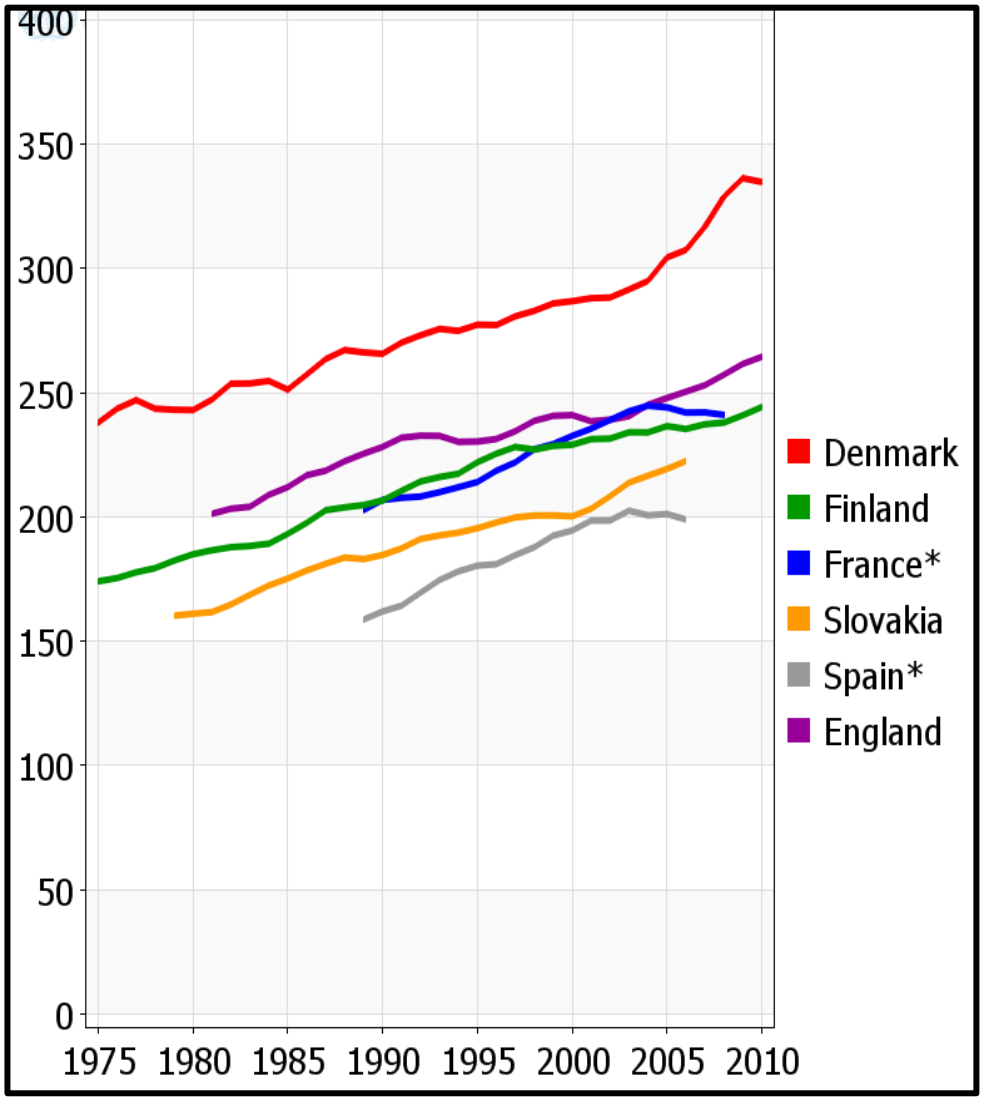

Figure 11: Cancer incidence trends in women selected countries: WHO - 1975 to 2010

Age-standardised rate per 100,000, women. The data shows a general trend in the increase of oral cancer; England having the second highest number of cancer incidences in comparison to the other countries shown.

* Regional data (NORDCAN – www.ancr.au; ECO – www.iarc.fr; ENGLAND – www.ons.gov.uk)

GLOBOCAN 2012 (IARC) (3.6.2015)
http://globocan.iarc.fr/old/FactSheets/cancers/All-tts-1i2.png

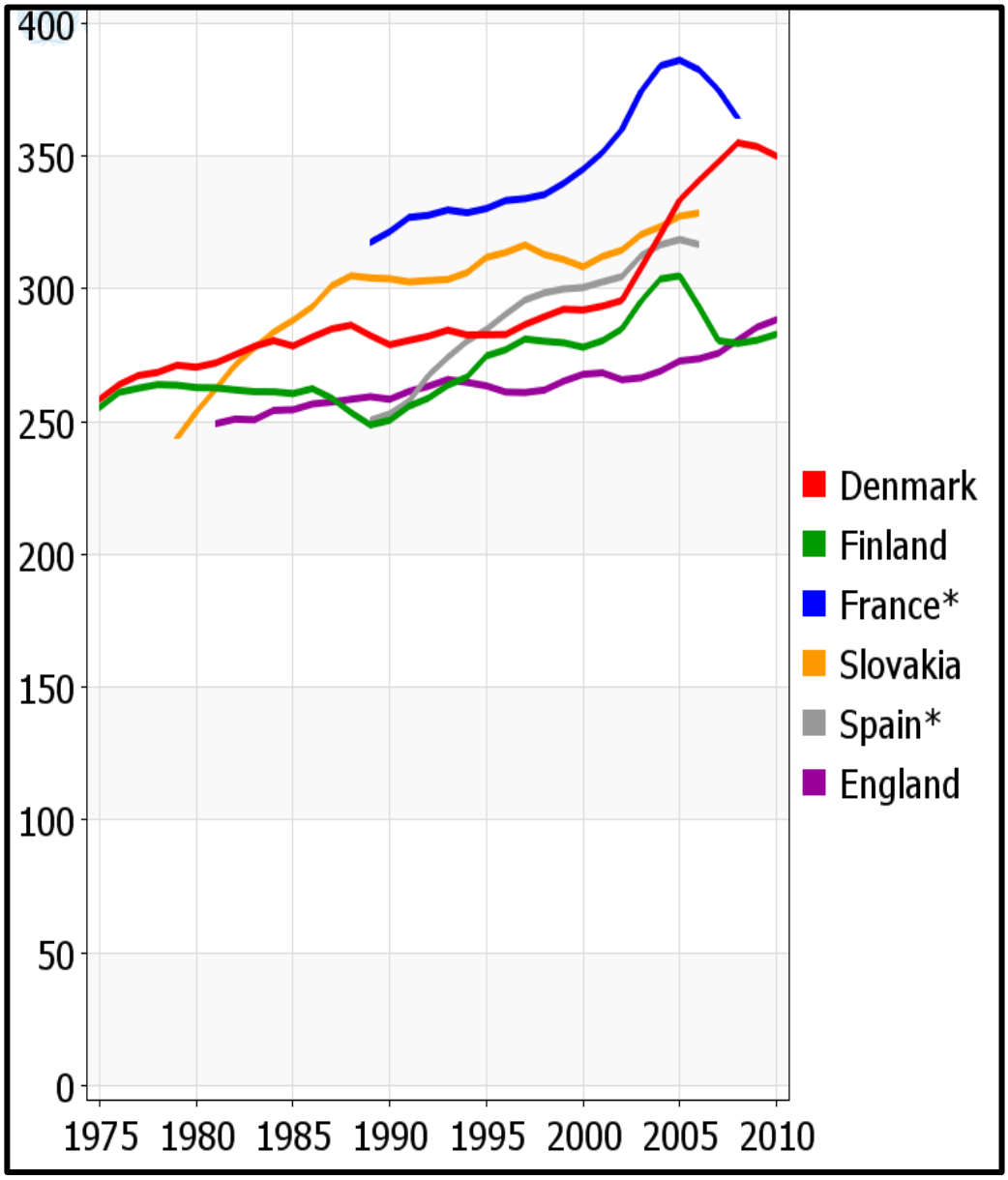

Figure 12: Cancer incidence trends in men in selected countries. Age-standardised rate per 100,000, men (WHO); 1975-2010.

The data shows a general trend in the increase of oral cancer; England having the second lowest number of cancer incidences in comparison to the other countries shown.

* Regional data (NORDCAN – www.ancr.au; ECO – www.iarc.fr; ENGLAND – www.ons.gov.uk)

GLOBOCAN 2012 (IARC) (3.6.2015)

http://globocan.iarc.fr/old/FactSheets/cancers/All-tts-1i1.png

Figure 11 and 12 above show trends in oral cancer incidences for both men and women in selected countries in Europe. These Figures show clearly the general increase in incidences for both male and females in all countries. For men in England, the incidence raised from 250 per 100,000 men in the early 1980's to around 290 cases per 100,000 in 2010. For women, a similar trend was observed, with an increase from 200 cases per 100,000 the early 1980's to about 270 cases per 100,000 women in 2010.

Appendix 5 shows incidence and prevalence of head and neck cancers in all of Europe for the 5 years leading to 2012; the incidences rates for head and neck cancer were shown to be gradually increased for most of the European countries. For example, the United Kingdom reported 11010 cases in 2007 and the number went up in 2010 by 234% before increasing further increasing in 2012 by 362%. A study has reported that HPV positive HNSCC are prevalent in whites and of a higher socioeconomic status (Young *et al*., 2015). World estimates databases on head and neck cancer, were searched for trends using gender and economic status of countries in the world, i.e. developed and less developed.

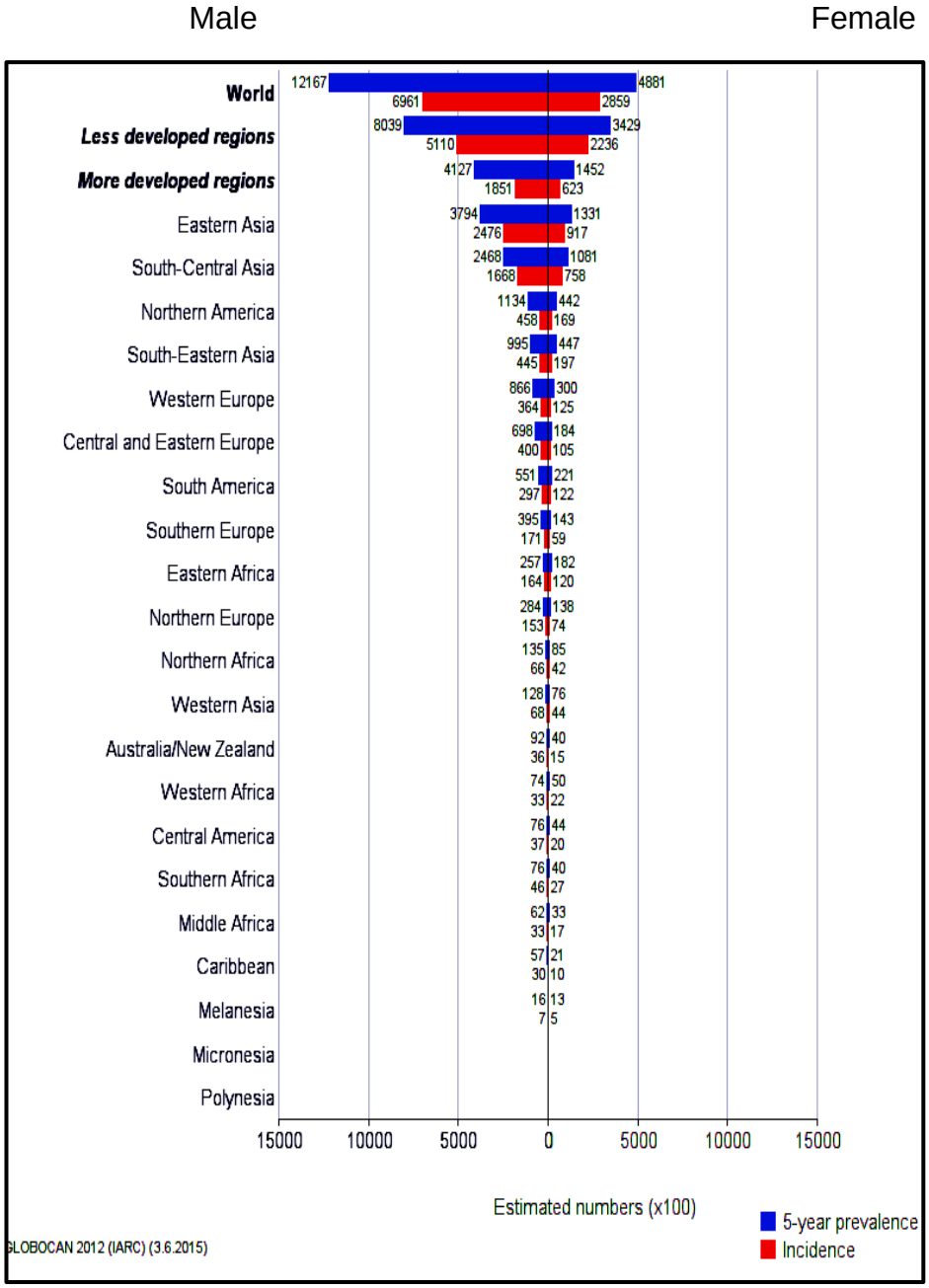

Figure 13: World estimates on head and neck cancer, using gender as well as the economic status of world countries.

World estimates on head and neck cancer i.e. developed compared to less developed countries for head and neck regions from 2007-2012

GLOBOCAN 2012 (IARC) (3.6.2015)
http://globocan.iarc.fr/data/GLOBOCAN_BSC_5910189.png

Figure 13 above shows GLOBOCAN world estimates on oral cancer incidences for developed and less developed countries, as well as the gender distribution of oral cancer in world regions. Worldwide oral cancer incidences are higher for males compared to females. Oral cancer incidence is higher in less developed countries compared to more developed countries. Figure 13 also shows most oral cancer cases as prevalent in the Asian countries where the use of betel nut is responsible for most of the oral cancer cases in those regions (Milgrom *et al*., 2016). The African continent has a very low prevalence rate of oral cancer incidences.

A significant increase of oral cancer cases not associated with betel nut use were reported in the developed countries mainly North America and Europe (Milgrom *et al*., 2016). Oral cancer incidences 20 European countries that have reported an increase in oral cancer incidences are shown in Figure 14. The United Kingdom is ranked number four in Europe per GLOBOCAN 2012 estimates with German at the top of the lists with a record number of 562 cases for males and 147 cases per 100,000 for women.

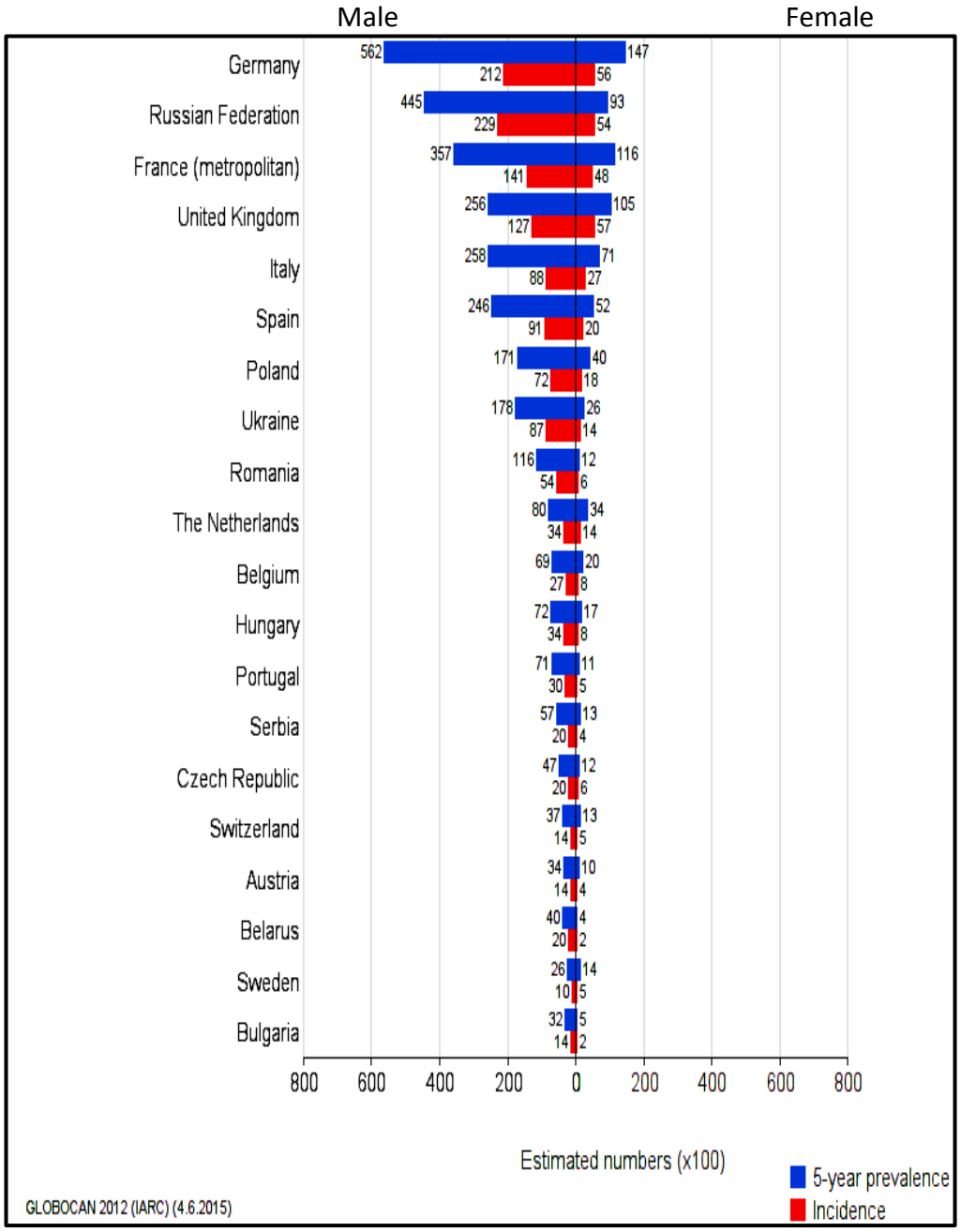

Figure 14: European estimates on head and neck cancer using gender.

The European estimates show the UK to have one of the highest incidences in Europe ranking fourth out of the 20 European countries shown.

(GLOBOCAN 2012); GLOBOCAN 2012 (IARC) (4.6.2015)

http://globocan.iarc.fr/data/GLOBOCAN_BSC_97063214.png

Figure 14 above shows the five-year prevalence and incidence of oral cancer in European countries. The Figure clearly shows German to have the highest number of oral cancer incidences with the UK ranked number 4 out of all European countries. Oral cancer incidence in Europe is increasing (Appendix 5). GLOBOCAN was used to predict the estimated number of new oral cancer cases in the UK by 2020 (see Figure 15). GLOBOCAN was used to estimate the number of new oral cancer cases in the year 2020 for all ages and both sexes in the UK and GLOBOCAN was utilized to predict the estimated number of new HNSCC cases in the UK by 2020 (see Figure 15). GLOBOCAN was used to produce an estimated number of new oral cancer cases in the year 2020 for all ages and both genders in the UK and results forecasted a rise in males of 14,552 and females 6.330 by 2020. Population forecasts were obtained from the World prospects 2012 update and the United Nations.

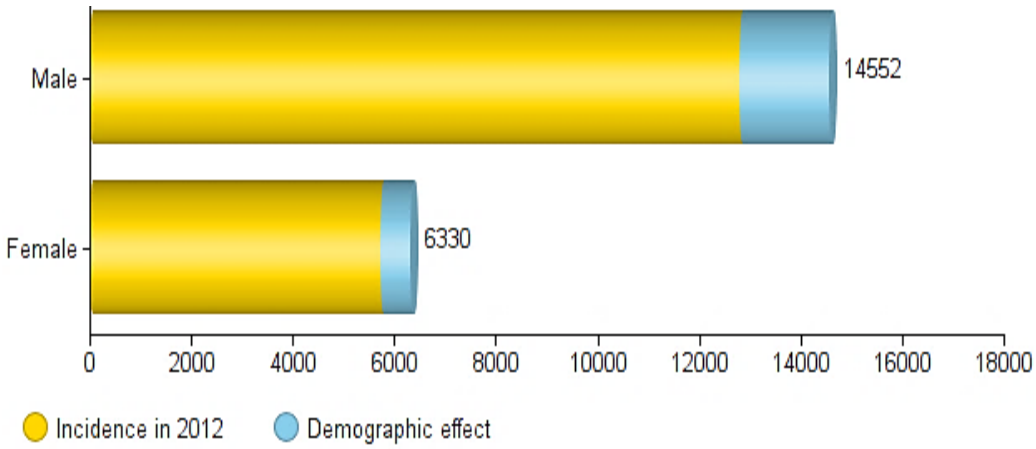

Figure 15: Predicted number of new oral cancer cases for both sexes in the year 2020 (UK) for all ages.

GLOBOCAN 2012 (IARC) (4.6.2015)

http://globocan.iarc.fr/data/GLOBOCAN2012_BURDEN_44899420MF.png

Figure 15 above demonstrates the GLOBOCAN predicted rise in HNSCC in the UK by the year 2020. The predicted rise is in line with other research that has shown a remarkable incidence in the numbers of HNSCC (Ferlay *et al*., 2015). The GLOBOCAN estimates shown forecast a sharp rise of oral cancer cases in both man and woman for the years 2012 to 2020.

4.2 UK Oral Cancer incidence trends

Oral cancer incidence trends in the UK where assessed using data from the Cancer Registry UK. Available data was the from year 1975 to 2011; see Figures 16, 17 and 18 below.

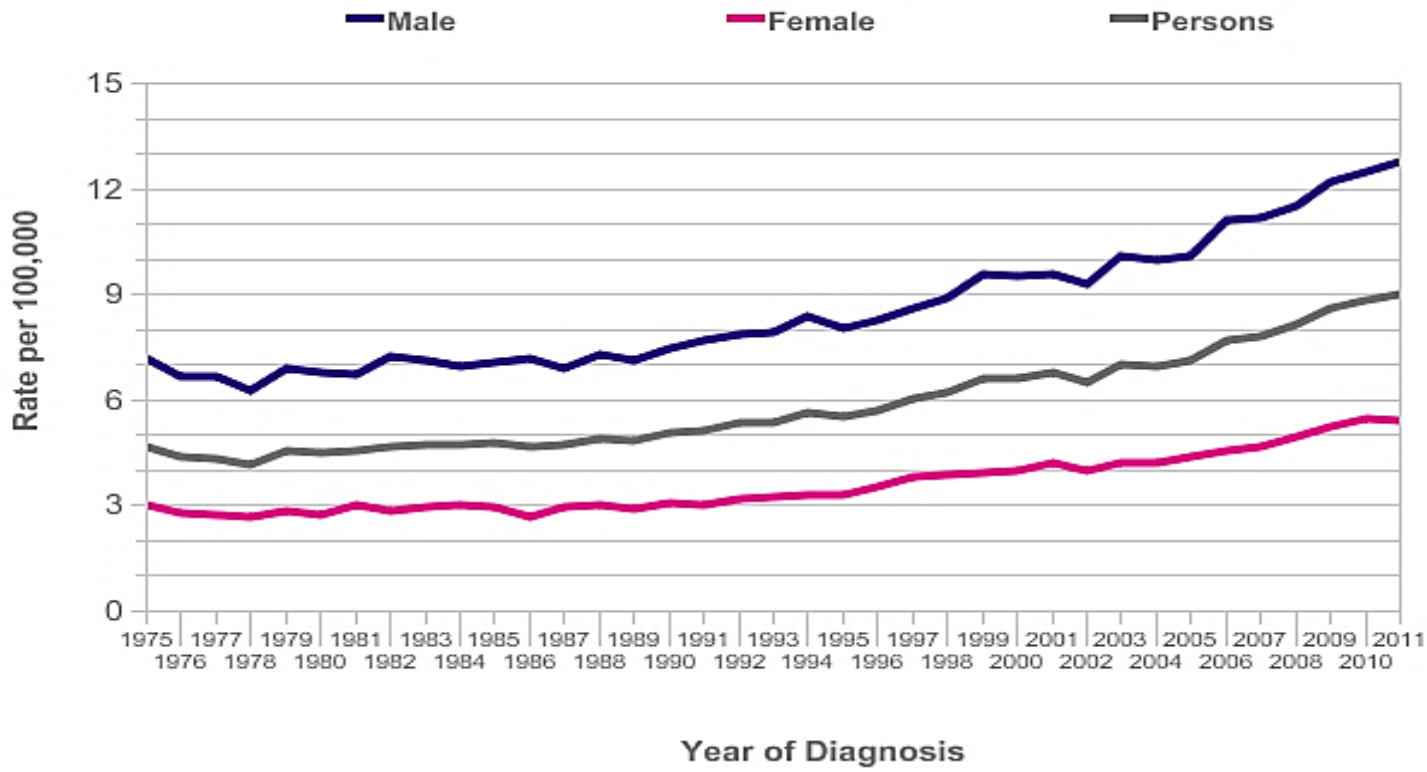

Figure 16: Great Britain; 1975-2011 - European Age-Standardized Incidence Rates by Sex per 100,000 Population

http://info.cancerresearchuk.org/cancerstats/faqs/#How; Cancer Research UK (15.06.2015)

HPV – known cause of cervical cancer, Increasing HNSCC incidence, change in sexual habits.

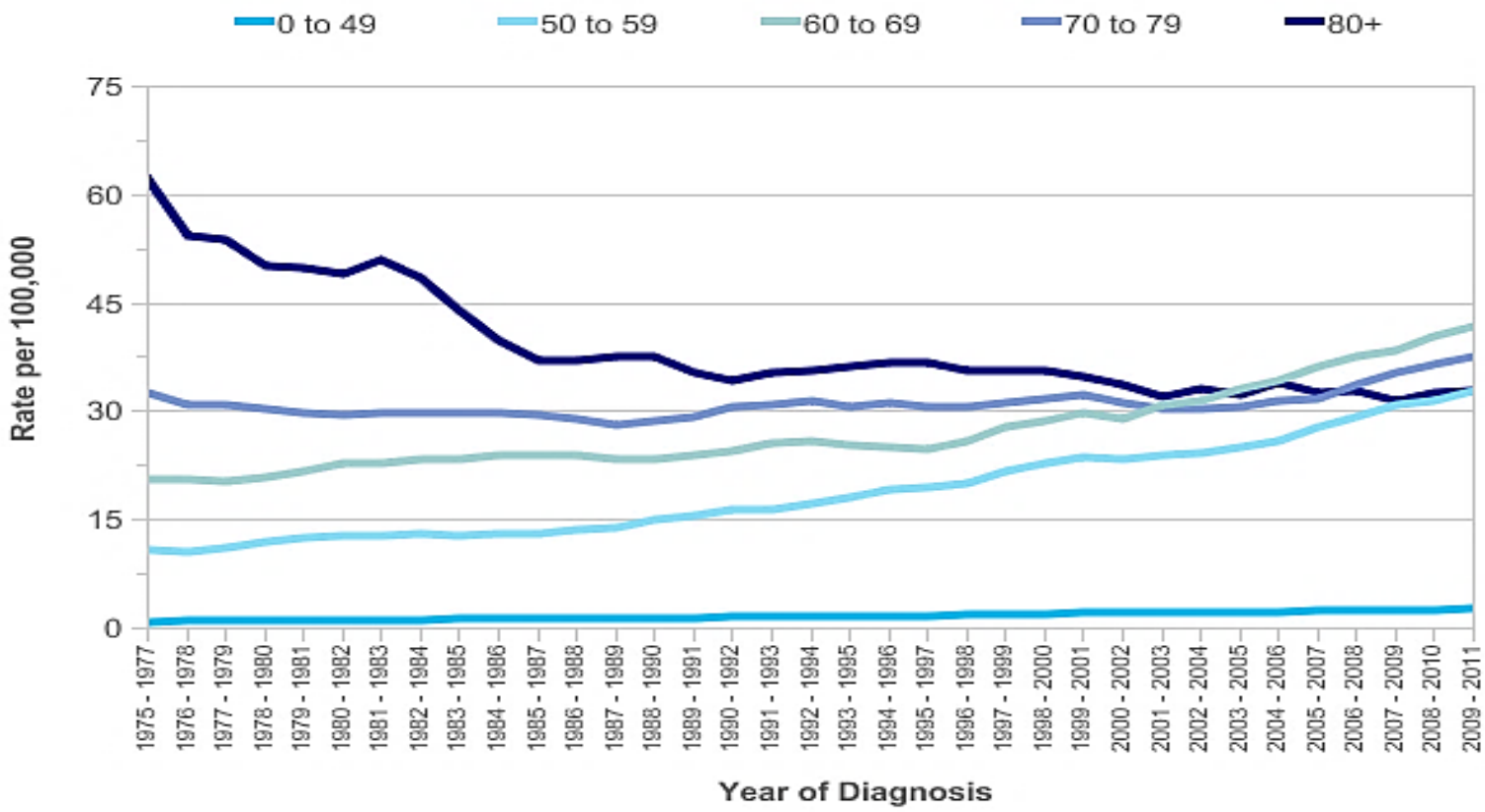

Figure 17: European Age-Standardised Incidence Rates for Males according to age per 100,000 Population, Great Britain; 1975-2011.

http://info.cancerresearchuk.org/cancerstats/faqs/#How; Cancer Research UK (15.06.2015)

HPV – known cause of cervical cancer, Increasing HNSCC incidence, change in sexual habits.

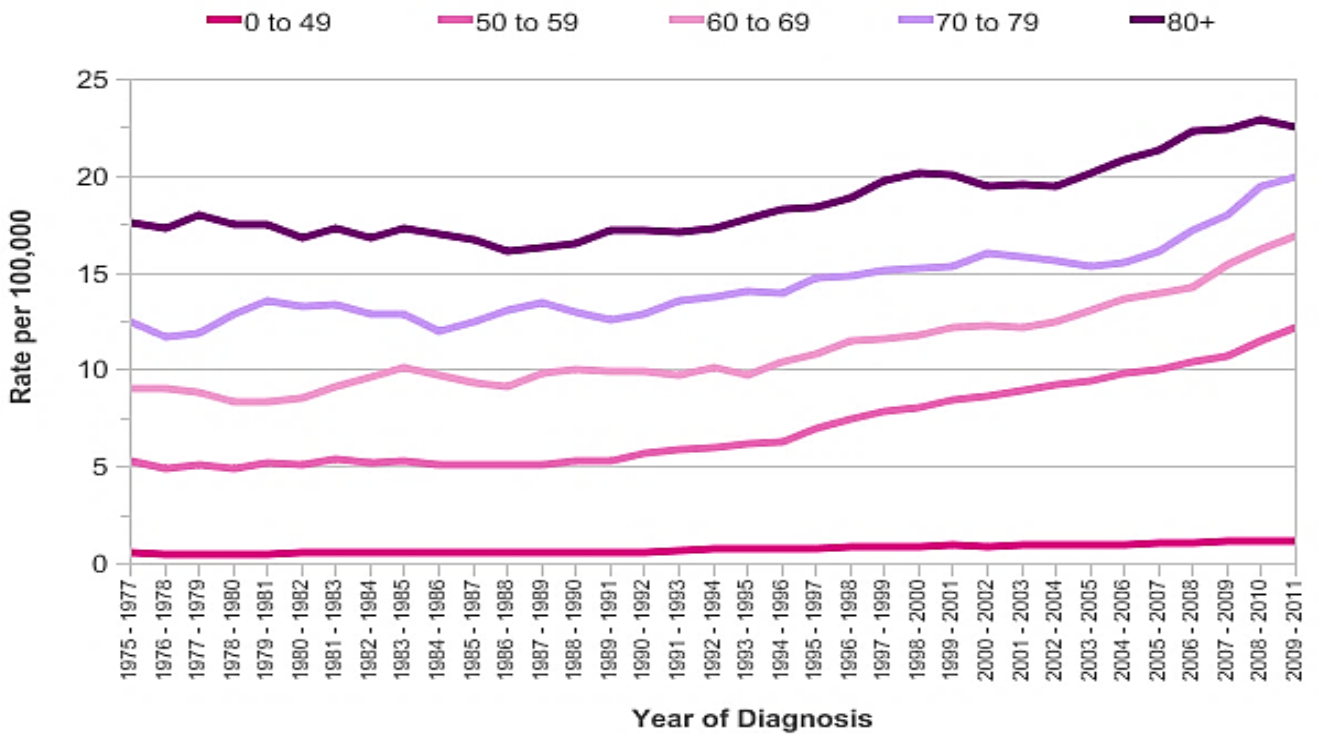

Figure 18: Female Age-Standardised Incidence Rates in Europe per 100,000 Population in relation to Age (Great Britain; 1975-2011).

http://info.cancerresearchuk.org/cancerstats/faqs/#How; Cancer Research UK (15.06.2015)

HPV – known cause of cervical cancer, Increasing HNSCC incidence, change in sexual habits.

Results shown in Figures 16, 17 and 18 above reveal an overall increase of oral cancer incidences in the UK from the mid-1970s for most age categories except males above 80 years. The 50-59 age specific age groups for males showed the largest increases with European age standardised incidences tripling (202%) in the years 1975-1977 as well as 2009-2011 (Cancer Research UK). A comparable trend was observed for females, where those 50-59 old reported highest increases, with rates doubling by 131% in the years 1975-1977 as well as 2009-2011 (Cancer Research UK). Comparative patterns for young and moderately aged individuals were reported in different regions of the world (Annertz *et al*., 2012; Patel, 2011; Shiboski *et al*., 2005; Golas *et al*., 2007). As indicated by Cancer Research UK, high HPV incidence rates are probably responsible for the increased frequency of some oral carcinomas among the relatively younger age groups (NICN Briefing 2012). The same observation was reported in the present study where most HPV positive patients were in the 50-59 age groups.

4.2.1 Oral cancer in the UK in relation to age

Data was collected from the Cancer Research UK to estimate the average number of people diagnosed yearly with oral cancer. The data used by the Cancer research UK to estimate the number of individuals diagnosed yearly with oral cancer was obtained from the Office for National Statistics, and other cancer registries across the UK. There is a strong relationship between oral

cancer incidence and age of participants as discussed in the introduction, though the patterns differ between genders.

The period 2009 to 2011 in the UK, in males (15%, 71%) of cases were diagnosed in those above ages 75 and those aged 50-74 respectively. For females, (29%, 59%) of cases were diagnosed in those above ages 75 and those aged 50-74 respectively (Cancer Research UK) (see Figure 19). The results are consistent with results from the present study where the mean age for diagnosis was 56.2 years for both male and females. Age specific incidences as per Cancer Research UK for men increased sharply from 40-44 years, with peak incidences in the 60-64 age groups.

A sharp rise in numbers was evident from the present research after the age of 44, however the peak age was below 60 years for both men and women. According to the Cancer Research UK, for females the pattern for age specific rates rose gradually from around 40-44, however in comparison to males the highest rate was in the 85 plus age group (See Figure 19). There were higher oral cancer incidences in men versus women with the widest gap in the 45-49-year age range where the male: female ratio for age specific rates was 28:10. Figure 19 below shows the yearly average new oral cancer cases and specific for all ages per 100,000 populations in the UK for both genders.

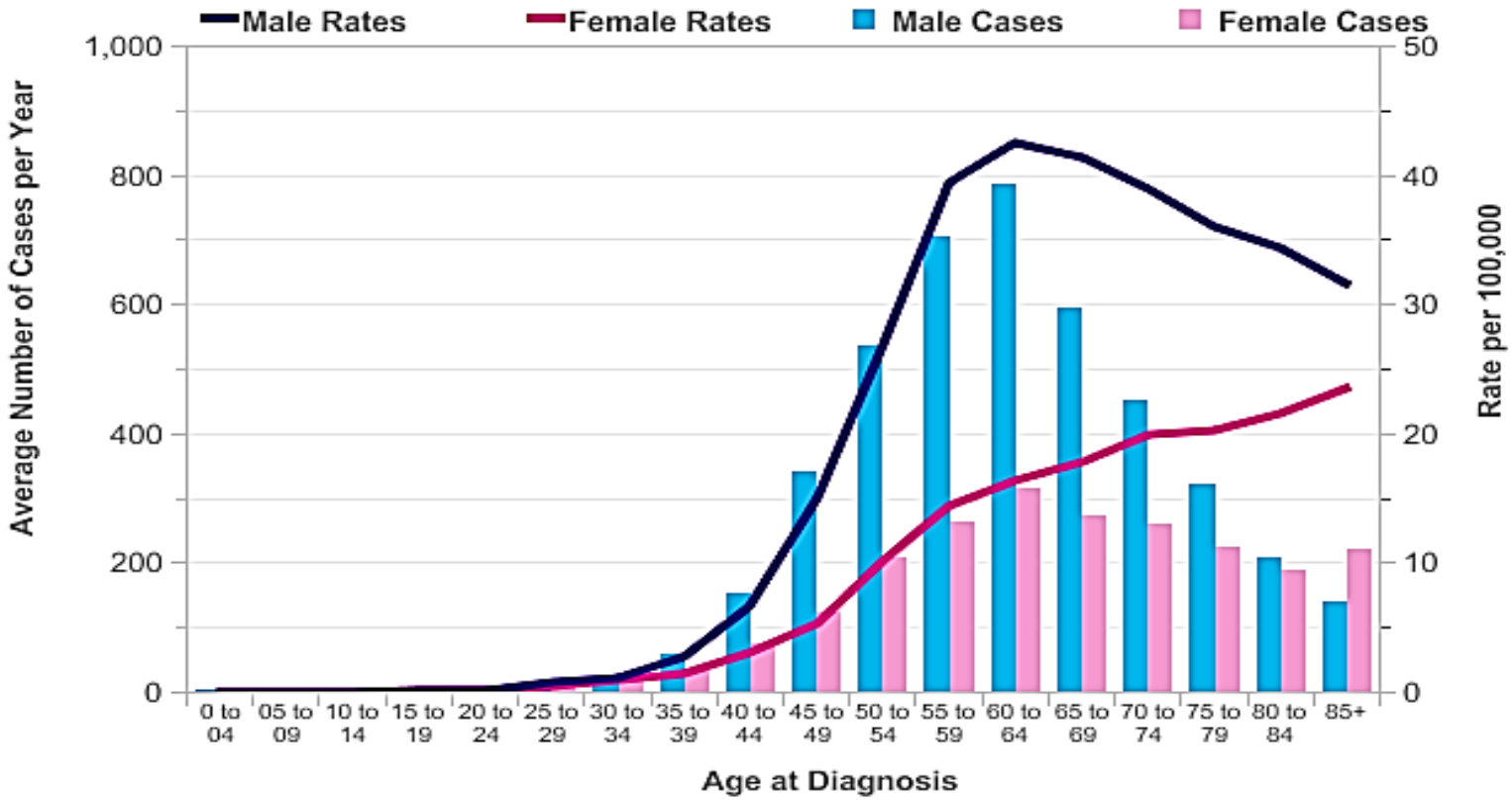

Figure 19: Male and female age specific incident rates and average numbers of new cases per year per 100,000 of the UK population, 2009-2011.

http://info.cancerresearchuk.org/cancerstats/faqs/#How; Cancer Research UK (15.06.2015)

Figure 19 shows oral cancer incidence rate in the UK for males to have increased from age 45 to 64, followed by a sharp decline after age 65.

HPV – known cause of cervical cancer, Increasing HNSCC incidence, change in sexual habits.

4.2.2 Incidence of oral cancer at regional level in the UK

The PHE website was used as a source of oral cancer incidences data at regional level in the UK. The results are shown in Table 15 below. The East midlands region of the UK was analysed for this research. Trends in oral cancer incidences in the East Midlands were similar to other regions in the UK.

Table 15: Oral cancer incidence in the East Midlands in the UK.

Area	Count
England	18,936
East Midlands region	1,687
Derby	90
Derbyshire	311
Leicester	121
Leicestershire	225
Lincolnshire	255
Northamptonshire	244
Nottingham	122
Nottinghamshire	308
Rutland	11

Source: PHE - London Knowledge and Intelligence Team (in view of information removed from National Cancer Registration Service's cancer registration framework ENCORE).

Table 15 shows oral cancer incidence in the East Midlands region of the UK. The region accounts for 1,687 of the 18,936 oral cancer cases in the UK.

Northamptonshire revealed 244 new cases for every year positioning fourth highest in the region on the total number of newly diagnosed oral cancer cases.

4.2.3 Distribution of Oral Cancer Cases in the UK by site

The oral cancer group incorporate many sites inside the head and neck region though most are in the mouth and tongue regions, which accounted for 60% of all cases in 2010 (National Cancer Statistics). The observations were similar to the results in the present study, where 61% of tested biopsies were from the tongue region and 12% from the mouth region, with an overall percentage of 73% for both sites in comparison to all the other sites. Lip cancer accounts for possibly 6% of all oral cancer cases (Cancer Research UK). Tumour of the lip represents around 6% of all oral malignancy cases (Cancer Research UK). Lip tumours have unique risk factors compared to other oral carcinomas, for example, nasal, parotid organ, middle ear, sinuses, and larynx. Lip tumours are related to ultraviolet radiation from daylight or sunbeds.

The IARC classifies HPV type 16 as an entity of cancers of the oral cavity such as pharynx, and HPV type 18 as a probable entity for oral malignancy. HPV 16 was implicated as a cause of oral carcinomas in 23 out of 25 biopsies tested for HPV in the present research. That accounted for 92% of all HPV positive biopsies in the present research. HPV 16 was identified as a cause of oral cancer in studies elsewhere (Marcu, 2016; Carlson, 2016). In the UK, IARC evaluated 8% oral cavity malignancies and 14% oropharyngeal tumours to relate to HPV infection. In addition, according to IARC a meta-analysis showed

that, in Europe, 73% oropharyngeal tumour cases were HPV positive, this proportion had increased over time. In Europe, 12% oral cavity, hypopharyngeal and laryngeal cancer cases were positive for HPV, showing no change over some time. Mouth, tongue and oropharyngeal malignancy risk were increased in individuals with a high number of past sexual partners (particularly oral sex partners), and early sexual experience. That data reflected the sexual route of HPV transmission (IARC; see Table 16 below). The tongue and mouth regions showed highest increases in cases of oral cavity cancer.

Table 16: Number of new cases of oral cancer, by subsite, UK, 2010.

Cancer site	Male		Female		Persons		Male: Female ratio
	Cases	%	Cases	%	Cases	%	
Lip	248	5.8	133	6	381	5.8	1.9
Tongue	1.336	31	692	31	2.028	31	1.9
Mouth	1.101	25.6	819	36.7	1.920	29.4	1.3
Oropharynx	1.065	24.7	391	17.5	1.456	22.3	2.7
Piriform Sinus	231	5.4	54	2.4	285	4.4	4.3
Hypopharynx	146	3.4	63	2.8	209	3.2	2.3
Other and Ill-Defined Sites	180	4.2	80	3.6	260	4	2.3

%. Represent the overall percentage in the set category

4.3 Oral cancer risk factors

To assess the risk factors for oral cancer data was extrapolated from the Cancer Research UK. Data from IARC was also assessed. Table 17 below shows the known risk factors associated with oral cancer in the UK. The risks include tobacco use, and the presence of HPV type 16. Risk factors for HPV positive and HPV negative oral cancers have been reported as being different (Beachler *et al*., 2014; Heck *et al*., 2010).

Increase in oral carcinomas has been attributed to HPV positive carcinomas reported to have different risk factors compared to HPV negative carcinomas (Carlson, 2016; Osazuwa-Peters *et al*., 2015). HPV related carcinomas' risk factors have been cited to include sexual lifestyle such age at first sexual encounter, engagement in oral sex, high number of sexual partners, socio-economic status and young age (Carlson, 2016). Non-HPV related oral carcinoma is mainly attributed to smoking and alcohol abuse, older age, chewing betel nut as considered in section 5.1. In the present research, most of the HPV positive biopsies were obtained from individuals with no history of excessive use of alcohol and tobacco consumption.

4.3.1 Head and Neck sub-site distribution of Oral Cancer Cases

The Cancer Research UK reported the oral cancers to consist of many sites within the head and neck region (Parkin, 2011; Mehanna *et al*., 2013; Heck *et al*., 2010). The most widely recognized were the tongue and mouth, which in 2010 represented 60% of cases. The data from Cancer Research UK is in line

with the present study where 61% of biopsies from HNSCC were from the tongue and mouth regions.

Lip cancer represents around 6% of oral cancers and has different risk factors compared to other oral cancers (Cancer Research UK). Lip growths have different risk factors to other oral tumours. Ultraviolet radiation from daylight or sunbeds are risks for lip cancer. Other HNSCC is associated with cancers of the salivary organs, parotid organ, nasopharynx, ear and larynx. Samples collected from all these sites except nasal cavity and salivary glands were analysed for this research. HPV type 16 as per IARC is an entity in oral, pharynx and tonsillar cancers, whilst HPV type 18 was classified as an entity in oral cavity carcinoma (IARC, 2012).

According to Parkin, (2011) it is evaluated that 8% of oral carcinomas and 14% of oropharyngeal carcinomas are connected to HPV infection in the UK. Mehanna *et al*. (2013) study reported that 73% of oropharyngeal cancer, 12% of larynx, oral cavity and hypopharynx cancers in Europe were positive for HPV. As indicated by Heck *et al*. (2010) oropharynx, tonsil, and tongue malignancy risk are increased in individuals reporting many past sexual partners (especially oral sex), young age at first sexual experience which mirrors the HPV sexual course of transmission.

Table 17: Risk Factors for Oral Cancer.

Increases risk (adequate confirmation)	May increase risk (limited evidence)	May decrease risk (limited evidence)
Alcohol excessive use (oral cavity)	Hydrochlorothiazide (lip)	Consumption of Non-starchy vegetables (not salted or cured)
Smoking Betel quid and tobacco (oral cavity)	Solar radiation exposure (lip)	Consumption of Fruits (not salted or cured)
Smoking Betel alone (oral cavity)	Human papillomavirus virus acquisition (oral cavity)	Consumption of Foods containing carotenoids
Human papillomavirus type 16 (oral cavity)	Radioiodine's, including Iodine-131 (salivary gland)	
Smokeless tobacco use (oral cavity)	Asbestos exposure (pharynx)	
All Tobacco smoking (oral cavity, other sites)	Mate drinking (pharynx)	
Exposure to X-radiation, gamma-radiation (salivary gland)	Printing processes exposure (pharynx)	
Epstein-Barr virus (nasopharynx)	Second-hand tobacco smoke (pharynx)	
Exposure to formalin (nasopharynx)		
Salted fish (nasopharynx)		

IARC and The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) classifications.

4.4 HPV Vaccination

Data in Table 18 show that the Northamptonshire Local authority to be above the average England vaccination uptake for all doses with the highest percentages of vaccine uptake in the Hertfordshire and The South Midlands region.

Table 18: Annual HPV vaccine coverage in England: 2013-14 (PHE). Cohort 11, School Year 8 girls (12-13 year olds).

HPV vaccine coverage data for 3 doses; first, second and third dose by Local Authority for the routine cohort at 31 August 2014

Locality	Total No. of girls in Cohort 11	Doses given 1st September 2013 to 31st August 2014					
		Dose one		Dose one and two		All three doses	
		Number	%	Number	%	Number	%
England	284793	259479	91.1	255651	89.8	246918	86.7
Local Authority Vaccine Coverage Range		(73.0 - 99.4)		(54.3 - 96.9)		(51.1 - 96.6)	
Hertfordshire And The South Midlands Area Team							
Bedford Local Authority	1011	956	94.6	942	93.2	912	90.2
Central Bedfordshire Local Authority	1314	1219	92.8	1214	92.4	1199	91.2
Hertfordshire Local Authority	7276	6476	89.0	6460	88.8	6218	85.5
Luton Local Authority	1224	1088	88.9	1085	88.6	1053	86.0
Milton Keynes Local Authority	1422	1283	90.2	1253	88.1	1201	84.5
Northamptonshire Local Authority	3898	3766	96.6	3723	95.5	3654	93.7

%. Represent the overall percentage in the set category.

The UK government introduced HPV vaccination in teenage girls between the ages of 12-13 since 2006. HPV vaccine is thought to reduce the number of HPV cervical cancers in the future though there is no research yet to verify that information. Table 18 above also shows annual 2014 HPV vaccine coverage by local authority. The Table specifically shows the East Midlands Local authorities in comparison to the rest of England. Northamptonshire is shown in the Table to have the highest vaccine uptake (93.7%) in the 12-13 year olds in comparison to other areas, such as Milton Keynes (84.5%). The assumption is that the HPV vaccine may possibly impact on the increasing number of HPV positive oral carcinomas (Saraiya *et al*., 2012; Berkowitz *et al*., 2015). This however, still requires more research to investigate that phenomenon.

4.5 Gender and Race considerations

There is overwhelming evidence of the gender differences associated with oral cancer. Generally, the number of males diagnosed with oral cancer is higher than their female counterparts (see Table 16 above). That phenomenon was confirmed in the present research with 67.7% of participants being of male gender. Table 16 additionally demonstrates differences in incidence rates of cancer in the diverse head and neck regions amongst males and females There has been a noted difference in peak age of diagnosis for males compared to females (see Figure 20 above). The age of diagnosis for males is usually lower than that for females. The same aspect was shown in the present research where the mean age for males was 55.5 and that for females was 57.8. There is

no consensus about the effect of gender on prognosis in HPV oral squamous cell carcinoma.

4.5.1 Cancer Occurrence and survival by Race/Ethnicity

Cancer occurrence and rates of death differ significantly among racial and ethnic groups. A study in America reported an increase in HNSCC of the tongue in white females between 1973 and 2010 with an annual percentage increase of 0.53 (Joseph *et al*., 2015). In the same research, the change appeared to be restricted to white women below 50 years and seems to have become more pronounced in the 1990s. On the other hand, for African Americans, the incidence has decreased. The relative survival estimates at 1, 5 and 10 years were 86%, 63% and 54% for white women, and 76%, 46% and 33% for African American women (Joseph *et al*., 2015). Better survival was significantly connected with certain factors, for example, more youthful age, marital status, receipt of surgical treatment and high financial status. Higher death rates from oral malignancy have been recorded in men from the Indian subcontinent in the UK, than in the indigenous UK population.

The relation between oral cancer mortality is strongly linked to socioeconomic status. In men, increased mortality from oral carcinoma in most countries including the UK, is mainly observed in socially disadvantaged groups (Scully and Bedi, 2000). Variables known to add to racial inconsistencies in mortality vary by cancer site and incorporate different exposure risks (Joseph *et al*., 2015). There has been reported dramatic difference in survival between black

and white patients with HNSCC overall, with black patients experiencing twice the mortality of whites. Recent evidence points to the higher prevalence of HPV positive HNSCC in whites as a major contributor to this disparity (Joseph *et al*., 2015). Exclusion of HPV positive HNSCC on calculating survival rate could drastically reduce the differences in mortality rates between whites and other races. Data for the survival of HNSCC in whites versus black patients is scarce, largely due to a reduced number of black patients with HPV-positive disease. More research is required for comparison the survival of HNSCC in black and white patients, and to explore the underlying noted differences.

4.6 Expected number of cancer deaths in the UK

Mortality trends on HNSCC have been reported to differ between HPV related and HPV unrelated HNSCC. Patients with HPV positive biopsies respond well to treatment and therefore show better clinical outcome in comparison to their HPV negative counterparts. HPV related carcinomas are usually diagnosed in individuals of a relatively young age in comparison to HPV negative carcinomas. Results from GLOBOCAN on the trends in mortality rates of males are shown in Figure 20 below. From 1950 to 2010, fewer younger males have demised due to HNSCC. The number of deaths for those above 80 decreased from 225 per 100,000 in 1950 to 160 per 100,000 in 2010. A similar trend was observed for 30-54 year olds and 55-79 year olds where there was a reduction of <10 deaths in 1950 and 2010 respectively.

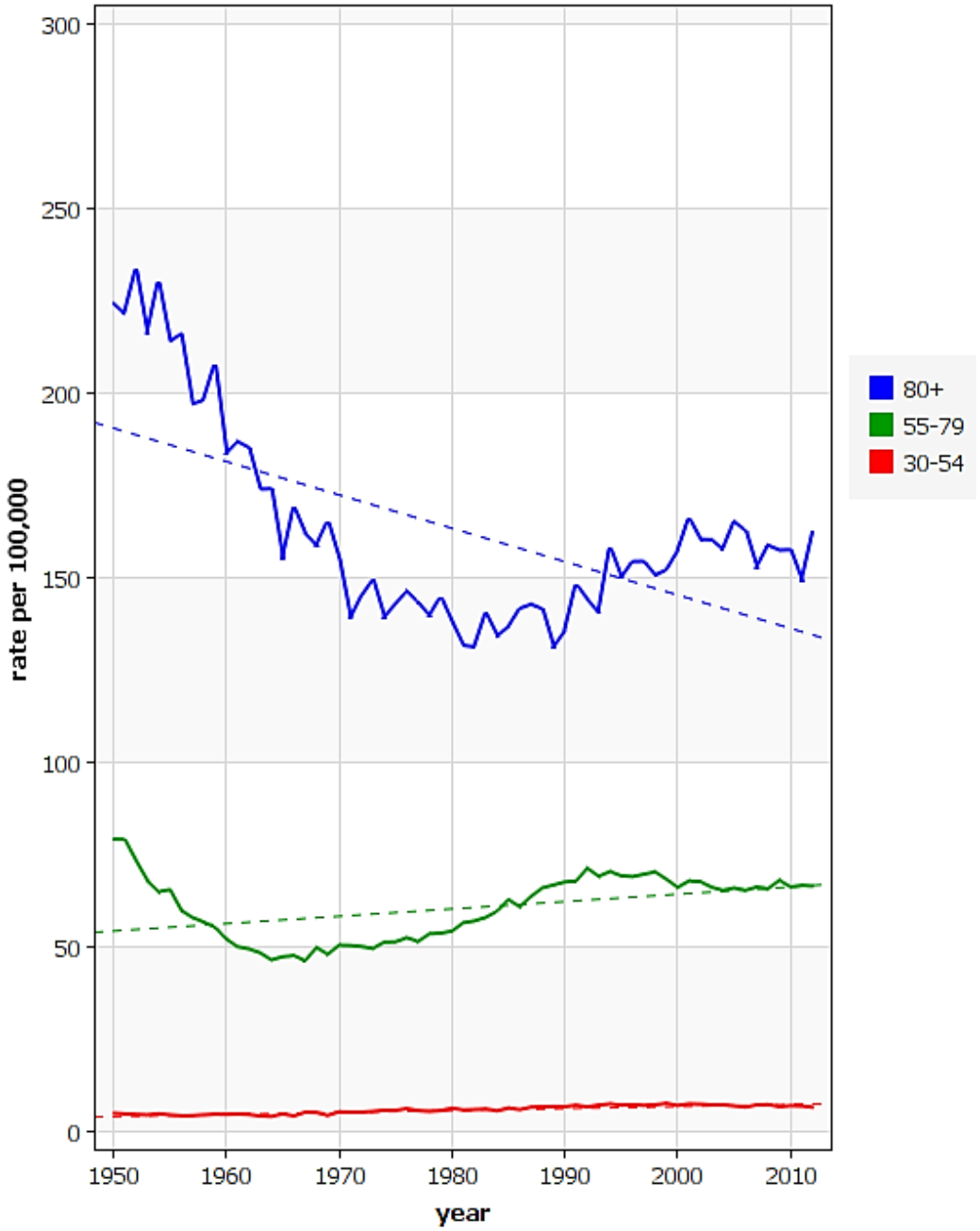

Figure 20: Male Mortality from HNSCC; UK, England and Wales, 1950-2010.

International Agency for Research on Cancer (IARC) (5.06.2015)
http://www-dep.iarc.fr/data/WHO_T2_46491351a.png

Figure 20 shows the mortality rate for males from HNSCC in the UK to be decreasing and similar trends were observed for females.

Chapter 5: Sexual Behaviour Results

5.1 Changes in sexual behaviour in the UK

HPV positive HNSCC has been linked to certain kinds of sexual behaviours such as multiple sexual partners. The noted change in sexual behaviour in the relatively younger population had been cited by some authors as the possible cause of increasing numbers of HPV related HNSCC (Rysavy *et al*., 2014; Osazuwa-Peters *et al*., 2015; Farsi *et al*., 2015). That phenomenon was investigated by analysing the trends in sexual behaviour in the UK. The behaviours analysed were the average number of sexual partners, same sex intercourse, oral sex trends, vaginal sex trends, age at first encounter and sexual health clinic attendance. The NATSAL website provided an insight into the sexual behaviour and attitudes in the UK.

The three NATSAL surveys undertaken in 1990-1991, 1999-2001 and 2010-2014 showed people having increased numbers of same sex partners and more people reporting high numbers of opposite sex partners. The data also showed that in the past decade there have been more increases of partners in women compared to man; therefore, the gender gap is narrowing. Table 19 below shows the results of the three NATSAL surveys. The results show a rise in the number of sexual partners for women compared to men. There were generally more sexual partners reported for men compared to women for NATSAL 1, 2 and 3; 8.6 *vs*. 3.7, 6.5 *vs*. 12.6 and 7.7 *vs*. 11.7 for men and women

respectively (NATSAL 2012). Increased number of sexual partners is a known risk factor for HPV related HNSCC (Farsi *et al*., 2015).

Table 19: Average number of sex partners in the UK (NATSAL UK).

Males average number of sex partners		Females average number of sex partners
8.6	Natsal-1 (1990-1991)	3.7
12.6	Natsal-2 (1999-2001)	6.5
11.7	Natsal-3 (2010-2012)	7.7

http://www.natsal.ac.uk/media/2102/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.16)

Table 19 above shows a general increase in the number of sex partners for both sexes. The numbers were shown to be on the increase, with higher numbers encountered increased gradually from NATSAL 1 survey to NATSAL 3 survey.

5.2 Average number of UK population with same sex experience

The numbers of people experiencing sex other than vaginal penetration have been cited to be on the increase. Same sex experience includes practices such as anal sex, oral sex, or other genital contact. Table 20 shows the percentage of the UK population that have experienced same sex or same sex including genital contact. NATSAL 1, 2 and 3 showed an increase of 4, 10 and 16% for females of the UK population respectively engaging in same sex experience, versus their male counterparts with 6, 8 and 7% respectively (NATSAL UK).

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Table 20: The NATSAL Average percentage of UK population with same sex experience.

	NATSAL 1 (1990-1991)		NATSAL 2 (1999-2001)		NATSAL 3 (2010-2012)	
	Males	Females	Males	Females	Males	Females
Percentage of population who have ever heard any same sex experience (16-44 years)	6%	4%	8%	10%	7%	16%
Percentage of population who have ever heard same sex with genital contact (16-44 years)	2%	4%	5%	5%	5%	8%

%. Represent the overall percentage in the set category

http://www.natsal.ac.uk/media/2102/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.16)

Table 20 shows the average number of individuals with same sex experience to be on the increase in the 16-44-year age groups with a fourfold increase in females in NATSAL 1 and NATSAL 3.

The above Table shows the percentage of people who have had same sex experience in all the three NATSAL surveys, NATSAL 1, 2 and 3. In the 16–44 age groups, there is general increase in females in the number of people who ever heard same sex experience or same sex with genital contact. That percentage for females increased from 4% to 16 % in 1990 and 2012 respectively. There was no difference for the past three decades in the number of males who ever heard same sex with an average of 7%.

5.2.1 Oral Sex: Different types of sex with people of same or opposite sex

Engagement in oral sex as has previously been cited by some studies is a major risk factor for HPV positive HNSCC (Rosenquist, 2016; Montero and Patel, 2015). There is a reported link between HPV HNSCC to changing life style choices such as having oral sex at an earlier age (Knight *et al*., 2016). A study by Farsi *et al*. (2015) reported higher number of oral sex partners as a huge risk factor for HPV elevated HNSCC. Individuals with several oral sex partners were reported by some studies having increased likelihood of oral HPV infection that is persistent (Kreimer *et al*., 2004; Kero *et al*., 2012). People with a higher number of lifetime and additionally recent vaginal sex partners are vulnerable to oral HPV infection, proposing that HPV is likely transmitted through oral sex (Fakhry and D'Souza, 2013). Numerous sexual behaviours are connected and usually individuals who have a high number of sexual partners for one act also have a higher number of the other acts (i.e. the important sexual exposures are collinear) (D'Souza *et al*., 2007; Smith *et al*., 2004).

Trends in oral sex behaviour in the UK among the different age groups were investigated (Table 21). The Table shows 70% of relatively younger males between 16 and 54 to have engaged in oral sex. In the 55-64 and 65 to 74 male age groups the percentage sharply decreased to 52%, and 30% respectively. A similar trend was observed in females with those aged between 16 and 54 showing 63% of that population engaging in oral sex. That percentage sharply dropped in those above 54, with only 35% of those aged between 55-64 engaging in oral sex and 19% of females in the 65-74 age group reporting oral sex experience.

Table 21: The average number of UK population engaging in oral sex for the specific age groups (NATSAL UK).

Males average (%)	Age at interview	Females average (%)
71	16-24	71
80	25-34	80
80	35-44	75
71	45-54	63
52	55-64	35
30	65-74	19

%. Represent the overall percentage in the set category.

The overall percentage of individuals engaging in oral sex is higher in the younger age groups for both sexes.

http://www.natsal.ac.uk/media/2102/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.16)

5.2.2 Anal Sex: different types of sex with people of opposite or same sex

Unlike oral sex, anal sex as a single entity, has not been reported as an important risk factor for HPV related HNSCC. Notwithstanding, people taking part in anal sex will probably take part in other sexual practices and have numerous sexual partners (Knight *et al*., 2016). Anal sex is also a risk factor for HPV related anal cancer (Fenkl *et al*., 2015). Data was sourced on anal sex trends in the UK from UK NATSAL. The results from the database are shown in Table 22 below. A maximum of 19% of the UK population reported to have had anal sex in their lifetime. In addition, anal sex was most common in the youngest age groups of 16 to 24 with 19 and 17% males and females in that age group having experienced anal sex.

The percentages gradually dropped as age increased with only 3 and 4 percent of males and females in the 65-74-year age groups having experienced anal sex. Anal HPV is normal and it is conceivable that oral-anal sexual contact (rimming) might be related to the transmission of oral HPV (Fakhry and D'Souza, 2013). One investigation of gay men demonstrated that a high number of rimming partners was related with higher oral HPV predominance, however additional research is required to clarify the role of rimming in oral HPV transmission (Beachler *et al*., 2012)

Table 22: The average percentage of UK population for the different age groups experiencing anal sex (NATSAL UK).

Anal sex		
Males average (%)	Age at interview	Females average (%)
19	16-24	17
17	25-34	16
15	35-44	13
14	45-54	8
8	55-64	4
3	65-74	4

%. Represent the overall percentage in the set category

http://www.natsal.ac.uk/media/2102/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.16)

5.2.3 Vaginal Sex

High numbers of lifetime oral sex partners (>5) and lifetime vaginal sex partners (>25) has been reported to be associated with higher risk of HPV positive HNSCC (Young *et al*., 2015). Data from NATSAL shown in Table 23 below showed at least 74% of the UK male population aged below 64 years to have experienced vaginal sex. Data on UK female population showed at least 59 percent of females aged below 64 years to have experienced vaginal sex. The highest numbers of individuals experiencing vaginal sex (91%) for both sexes were in the 35-44 and 25-34 age groups for males and females respectively. Percentages of the UK population experiencing vaginal sex were highest from 25-54 years for both males and females with a minimum of 81 percent and a maximum of 91 percent vaginal sex experience for both sexes. It is of interest to note that it is in the above age groups that HPV HNSCC has been reported to be more prevalent (Beachler *et al*., 2014). The results from the present research showed most HPV positive biopsies to be present in individuals below age 56.

Table 23: The average percentage of the UK population for the different age groups experiencing vaginal sex (NATSAL UK).

Vaginal sex		
Males average (%)	Age at interview	Females average (%)
74	16-24	75
89	25-34	91
91	35-44	89
85	45-54	81
75	55-64	59
57	65-74	37

%. Represent the overall percentage in the set category

http://www.natsal.ac.uk/media/2102/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.16)

Table 23 above shows NATSAL results for UK vaginal sex tends. From the Table, the data show many of the UK population to have experienced vaginal sex. A high number of vaginal sex partners has been cited as a risk factor for HNSCC (Young *et al*., 2015).

5.3 Age at first sexual encounter; UK trends

The age at which an individual first experiences sex has been reported as one of the risk factors for HPV associated HNSCC. Initial first sexual encounter at a younger age has been associated with increased engagement in risky sexual behaviour and a possibility of having several sexual partners. In a study in China, younger women who experienced sex at a younger age would probably have more sexual partners contrasted to their older counterparts (Zhao *et al*., 2012). Moreover, that research showed a trend towards earlier sexual encounter and high-risk sexual behaviours especially associated with younger Chinese women.

That had vital implications for the health of pre-adult young woman because of the relationship of youthful age at first sexual experience with the consequent medical issues and subsequent risky sexual conduct (Tilahun, and Ayele, 2013). Samek *et al.* (2014) research revealed many factors related with youthful age at first experience for example, low financial status, limited education, separated parents, living with a partner, having no religion, tobacco use, and drug use. Olesen *et al.* (2012) studied young age and risk taking behaviour in Nordic countries. Their study reported that there was a relationship of youthful age at first sexual experience with smoking and high alcohol consumption. Certain tendencies, for example, excessive alcohol consumption before sexual relations were related with unsafe sex due to improper use of condoms (Farid *et al*., 2013; Hugo *et al*., 2011; Tilahun and Ayele, 2013). The median age of

sexual intercourse sourced from NATSAL data in the UK population from 1935 are shown in Table 24. From the Table, the median age of first sexual intercourse has gradually reduced with records from 1935 stating median age of 18 for both sexes and records in 2012 reporting median ages of sexual intercourse as low as 15.5 for both sexes for those born in 1990-1996. UK NATSAL survey results for the percentage of the UK population engaging in sex before age of 16 are shown in Table 24.

Table 24: The median age at first intercourse (NATSAL UK).

Year of birth	Approx. Median age of first intercourse	
	Males	Females
1935-1939	18	19.5
1940-1944	18	19
1945-1949	18	18
1950-1954	17	17
1955-1959	17	17
1960-1964	17	17
1965-1969	17	17
1970-1974	17	17
1975-1979	17	17
1980-1984	17	17
1985-1989	17	15.5
1990-1996	15.5	15.5

Table 24 above shows the average age at first sexual debut in the UK population for both sexes dating back from 1935 to 1996. Overall, the age of sexual debut is decreasing in the UK population. Table 25 below shows NATSAL survey results for the average percentage of the UK population having sexual contact with someone of the opposite sex before the age of 16.

Table 25: Percentage of UK population who had sexual intercourse with someone opposite sex before the age 16 (NATSAL 2012 UK)

Percentage who had sexual intercourse with someone of the opposite sex before age 16		
Males average	Age at interview	Females average
31	16-24	29
26	25-34	25
27	35-44	18
27	45-54	15
17	55-64	10
15	65-74	4

http://www.natsal.ac.uk/media/2012/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.16)

The data shows the trend to be changing with younger age groups having sex with someone of the opposite sex before the age of 16; highest numbers are in the 16-24 year olds.

Table 25 above shows the number of people having sex before age 16 to be increasing over the past three decades. The 65-74 female age group shows a 4% average compared to the 29% average shown in the 16-24 age group of the surveyed female population. A similar increase was noted for the male population, with 15% in the 65-75 age group engaging in sex before the age of 16 in comparison to 31% in the 16-24 age group. In general, there is an increase in sexual activity before the age of 16 in younger people compared to older people in the population. The NATSAL surveys results showed the national average for age at first sexual encounter to be reduced for both sexes showing that increased numbers of people are engaging in sex before the age of 16.

5.4 Sexual health clinic attendance trends

Generally, people attending the SHC exhibit risky sexual behaviours which are likely to lead to sexually transmitted diseases that include HPV infection in various sites in the body. The patients have increased the likelihood of higher numbers of lifetime partners and to engage in more than one form of sexual activity. The 16-44-year age group UK NASTAL survey results are shown Table 26 below, for that age group, there is a general increase in sexual partners. Results from the 1990-1992 NATSAL survey showed that there was an average of 5% in the number of people between 16-44 years' age groups that had 2-4 partners in the five previous years before that survey that attended the SHC. That number increased to 7 and 9 (1999-2001) and 23 and 31 (2010-2012) for

males and females respectively. The percentage of the UK population attending the SHC increased as the number of sexual partners increased. The category for those with 5-9 partners showed an increase of (12, 10, 33%: 15, 17, 30%) respectively for males and females in the NATSAL surveys 1, 2, and 3 respectively. Finally, (20, 25 and 55%: 15, 55 and 65%) males and females respectively with more than 10 partners attended the SHC during the periods of NASTAL 1, 2 and 3 surveys, respectively. Therefore, Table 26 generally shows that the increased likelihood of attending the SHC is directly proportional to increased numbers of sexual partners. Furthermore, observed increase of people attending the SHC in the last NATSAL survey indicates that more people are having multiple partners in comparison to previous years.

Table 26: Sexual health clinic attendance, past 5 years (people aged 16–44) (NATSAL UK).

	NASTAL 1 (1990-1991)		NASTAL 2 (1999-2001)		NATSAL 3 (2010-2012)	
	Males (%)	Females (%)	Males (%)	Females (%)	Males (%)	Females (%)
Number of partners, past 5 years (0-1)	1	1	2	4	4	4
Number of partners, past 5 years (2-4)	5	5	7	9	23	31
Number of partners, past 5 years (5-9)	12	15	10	17	33	30
Number of partners, past 5 years (10+)	20	15	25	55	55	65

%. Represent the overall percentage in the set category

http: //www.natsal.ac.uk/media/2102/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.16)

The data shows that the more the number of sexual partners the more the likelihood of attending the SHC.

Table 26 above shows the increased numbers of the UK population attending the SHC. Attendance of the SHC was highly connected with increased numbers of sexual partners. Increased numbers of sexual partners increase the risk of sexually transmitted diseases. One of the other diseases identified with risky sexual behaviour is autoimmune deficiency syndrome (AIDS) which emanate from infection with the human immunodeficiency virus (HIV). Table 27 below shows the average number of the UK population that were tested for HIV (16-44) in relationship to the number of partners they had encountered in a five-year period. The Table plainly demonstrates that the more the number of lifetime sexual partners the more you were probably going to be tested for HIV.

The highest percentages of individuals tested for HIV are in the NASTAL 3 2012 survey where (6, 18.5; 14, 28; 25, 45; 43, 50) males and females with (0-1; 2-4; 5-9; 10+) respectively were tested for HIV. That is a substantial increase compared to NASTAL 1 1990 survey where (2, 9: 6, 13; 13, 19; 20, 25) percent of males and females with (0-1; 2-4; 5-9; 10+) respectively were tested for HIV. Therefore, the more the number of sexual partners the more likely you are to be exposed to infection with HIV. Individuals infected with HIV are more prone to HPV infection in comparison to HIV negative individuals. Furthermore, people that acquire HIV infection also have the same chance of acquiring HPV infection, both HIV and HPV HNSCC are sexually transmitted diseases acquired due to sexually risky behaviour. HIV infection have been associated by

research as a significant risk factor for acquiring HPV HNSCC (Beachler *et al*., 2014; Goncalves *et al*., 2016).

Table 27: HIV testing, past 5 years (people aged 16–44) (NATSAL UK).

	NASTAL 1 (1990-1991)		NASTAL 2 (1999-2001)		NATSAL 3 (2010-2012)	
	Males (%)	Females (%)	Males (%)	Females (%)	Males (%)	Females (%)
Number of partners, past 5 years (0-1)	2	9	4	7.5	6	18.5
Number of partners, past 5 years (2-4)	6	13	9	11	14	28
Number of partners, past 5 years (5-9)	13	19	14	17	25	45
Number of partners, past 5 years (10+)	20	25	22	22	43	50

%. Represent the overall percentage in the set category

http://www.natsal.ac.uk/media/2102/natsal-infographic.pdf

http://www.natsal.ac.uk/home.aspx (08.06.15)

The data shows that the more the number of partners the more the likelihood of being tested for HIV infecti

Chapter 6: Discussion – Oral Cancer Biopsies

The epidemiology of head and neck cancer has dramatically changed over the past four decades. Tobacco use, with or without alcohol abuse are historically known risk factors for HNSCC. Laws on smoking have significantly reduced the number of smokers. The prevalence of tobacco related HPV negative cancers has also decreased. In sharp contrast, the numbers of HPV related cancers have been steadily rising in the younger age group of below the age of 55 (D'Souza and Dempse, 2011). Data from Sweden and USA have revealed a remarkable increase in oropharyngeal cancers attributed to HPV (Nasman *et al*., 2009; Montero and Patel, 2015).

The incidence of HPV and non-HPV associated HNSCC is interestingly twice higher in males compared to females. Increased tobacco use and alcohol use is thought to drive the higher rates in non-HPV associated cancers. The 2-fold increase in HPV associated cancers witnessed more in men than women is still not understood. According to D'Souza and Dempsey (2011) one hypothesis is that the HPV burden could be higher in the vagina than the penis, with males acquiring HPV during oral sex to a female. Research by Hernandez *et al*. (2008) showed HPV transmission during sex to be mainly from an infected cervix to the penis than vice-versa, therefore supporting the above hypothesis.

There has been an increase in HPV associated HNSCC since the mid-1980s when HPV HNSCC was first reported. Rising rates of oral cancer in the base of the tongue, tonsil and mouth have been observed in Europe and the USA

(Hammarstedt *et al*., 2006; Chaturvedi *et al*., 2011). In the USA, from 1984 to 2004 the incidence rates of HPV-associated ORCC rose from 0.8 to 2.6 per 100,000 persons per annum, i.e. from 16.3% of HPV positive cases to 71.7% over that period (Chaturvedi *et al*., 2011). There was a 3-fold rise in HPV associated tonsil cancer between 1970 (29%) and 2007 (93%) and a 2-fold increase between 1956 and 2000 in Sweden and Finland (Syrjanen, 2004; Nasman *et al*., 2009). A 3-fold increase was similarly observed in England between 1995 and 2010 (Palmer *et al*., 2014).

Significant increases were noted in Australia, where HPV positive oropharyngeal, tongue, tonsil and mouth cancer increased from 20.2% in 1987–1995 to 63.5% in the 2006–2010 periods (Hong *et al*., 2014). Data from North America reported 69.7% prevalence of HPV HNSCC and data from South America and Europe reported 73.1% in those regions (Mehanna *et al*., 2013). In the Asia Pacific region, lower prevalence rates were reported by some studies, with much lower proportions averaged for the association of HPV with ORCC (Kumar *et al*., 2003; Bahl *et al*., 2013; Shaikh *et al*., 2015). The Indian subcontinent, head and neck cancer accounts for 45% of all malignancies (Stell and Maran, 2012). This high number of oral cancer cases in that subcontinent is attributed to betel leaf, lime, catechu, or areca nut use common in that region (Sturgis, 2004).

6.1 HPV genotypes and their prognostic significance in HNSCC

HPV is a known aetiological agent of anogenital carcinoma. HPV as a causal aetiology in HNSCC is still a subject of debate. The present research tested for the presence of HPV in 99 patients with squamous cell carcinoma treated at Northampton General Hospital between the years of 2006-2014. The results revealed 25.2% of the biopsies to be positive for HPV, with 23 (96%) out of the 25 biopsies positive for HPV genotype 16. Garcı´a-de Marcos *et al*. (2014) have reported that the frequency of positive HPV biopsies in the head and neck regions is varied, ranging from 0-100%. The variation frequency has been attributed to the use of a different range of detection methods, specimen types, storage conditions, different geographic regions and population selection.

PCR, one of the gold standard methods for HPV genotyping, was in the present study used for analysis and detection of HPV in patient biopsies. Results from the present research are in line with research published elsewhere. Syrjänen *et al*. (2011) reported HPV prevalence of 36% for oropharyngeal, 23.5–33.7% for oral and 24% for laryngeal cancers using PCR. Similar results were reported in a meta-analysis undertaken on the impact of HPV on the risk of oral cancer and overall survival in HNSCC patients (Dayyani *et al*, 2010). Furthermore, other review studies and meta-analyses estimate the number of oral cancer attributed to HPV and HPV type 16 to be in the region of 25 - 30% (Sand and Jalouli, 2014). However, a UK cohort study of 142 biopsies showed conflicting results to the present study (Lopes *et al*., 2011). The research tested for the presence of HPV in oral biopsies and reported less than 2% of the biopsies to harbour

HPV, leading to the conclusion that oral cavity rarely harbours oncogenic HPV. In another UK based study, 87 biopsies form the head and neck regions showed an overall HPV prevalence of 14% (Heath *et al*., 2012). However, in that study most of the patients had a history of smoking and the mean age of diagnosis was 60 for HPV positive cases. Studies elsewhere report HPV HNSCC as being prevalent in those below ages 55 and with no history of smoking (Shaikh *et al*., 2015: Allison and Maleki 2016).

The present study was in line with those studies reporting a mean age at 56.2 for HPV positive cases. Furthermore, most HPV positive patients (88.9%) had no history of smoking. Those results from Asia Pacific, USA and UK, show that there is a growing trend in different parts of the world of HPV associated HNSCC in relatively young adults with no smoking history. That observation suggestions that HPV positive carcinomas in those of a younger age group are being caused by other risk factors other than smoking and history. There was a noticeable change in sexual habits in the past 40 years in the UK and different sexual trends were reported in those above and below 55 years of age in data collected in the NASTAL surveys 1, 2 and 3 for this research. Statistically significant results revealed a possible connection between HPV positivity and SHC attendance. Therefore, it is likely that change in sexual habits such as reported in the present research could possibly be connected to the increased numbers of HPV positive HNSCC in the UK and other countries.

Previous studies carried out on the subject reported the presence of HPV genotypes 16, 18 and 33 as leading causes of HNSCC (De Villiers *et al*., 1985; Attner *et al*., 2010; Koskinen *et al*., 2007; Liang *et al*., 2008; Tezal *et al*., 2009). The present study was in line with the above research with 92% of the HPV positive biopsies classified as HPV genotype 16. Only one specimen was positive for genotype 33 and the other specimen could not be successfully genotyped. A Spanish study showed HPV 16 (42.1%) to be the main genotype frequently isolated in HNSCC, followed by HPV 18 (26.3%), HPV 6 (10.5%) and others (Garcı´a-de Marcos *et al*., 2014). HPV 16 genotype is responsible for a vast number of cervical cancers in the UK, Europe and worldwide. Recently it was suggested that it also causes cancer in other oral regions.

The present study showed HPV 16 to be the main genotype frequently encountered in tumours of the head and neck. The results were in line with other studies (Garcı´a-de Marcos *et al*., 2014; Ruatava *et al*., 2012). Moreover, as per Rautava *et al*. (2012), HPV16 is the most common genotype in HNSCC but HPV6 and HPV11 can also be found in a minority of cancers, implying that these low-risk HPV-types are not entirely benign in the head and neck region. A study by Rautava *et al*. (2012) showed multiple-type infection to be the most common type of infections (45%), then followed by single type infection of HPV type 16 (35%). In that study, HPV 6 and HPV 11 were detected in 27.4% of the samples. For multiple-type-infections, 2–7 different genotypes were identified, with the most common genotypes in descending order being 16, 11, 18 and 6. However, HPV16 was present in 70.4% of the multiple-type infections.

The present research was carried primarily on oral specimens which consisted of 61% tongue, 12% mouth and 27% others samples that included laryngeal samples. The present study showed similar results with most of HPV positive samples identified from the tongue 19 (76%) followed by the mouth region 3 (12%). HPV was detected in 28.4% and 18.7% of the male and female cases respectively. Genotype distribution was similar for males and females with most cases positive for HPV genotype 16, and only case genotype 33 positive and another case with one unknown HPV genotype reported. HPV genotype 16 is a leading oncogenic HPV genotype that is a known entity in cervical cancer. HPV type16 have also been shown to cause cancer in other regions including anal and oral regions. It is still debatable as to how HPV is transmitted and persists in those regions, and the mechanisms allowing for oncogenic HPV to establish are still not clear. Studies to date have documented the presence of HPV in the oral flora as not an indication of the likelihood of cancer, as most HPV infections are cleared within a year of contact my natural immune mechanisms (D'Souza, 2012; Monsjou *et al*. 2013).

Previous research studies have suggested possible sexual transmission of HPV to the oral regions of the head and neck sites (Mork *et al*. 2001; Joseph and D'Souza, 2012; Monsjou *et al*. 2013). The above research studies reviewed data on patients that included age and sexual history. Results obtained suggested sexual transmission due to the high number of newly diagnosed patients being of a younger age with no history of smoking and alcohol abuse, which was previously known as the risk factors for oral carcinoma. In addition,

the sexual habits in most developed regions worldwide including UK, USA and other European countries have dramatically changed over the past 50 years with a large part of the population engaging in oral and anal sex. HPV genotyping results for the present study (though it was only carried out in one region of the UK) and statistical analysis from publicly available websites in the UK collected for the present research also suggests a significant change in sexual habits. More than half of biopsies tested were from patients below the 56 years of age. Statistical information on sexual habits carried out in the UK, reveal a dramatic shift over the past 40 years of change in sexual habits with a greater proportion of individuals under the age of 60 having engaged in oral sex. Furthermore, the numbers of sexual partners encountered over this period have increased, which could also be related to the noticed reduction in the age of first sexual debut from about 19 years of age 40 years ago, to about 15 years in the 2012 NATSAL survey for both males and females.

The present study showed a higher proportion of cases presenting from oral tongue and mouth regions compared to those presenting from pharyngeal and other sites. This could be attributed to a low number of samples collected and the criteria used for sample collection in the present study. In addition, there was a correlation between younger age and infection with HPV 16 genotype, however and there was no evident correlation between HR-HPV and tobacco smoking or alcohol intake. The present study did not further analyse the survival rate of HPV positive in comparison to HPV negative patients, but other research has suggested an improved survival rate for patients with HPV associated

HNSCC (Zhao *et al*. 2009; Joo *et al*. 2012). Given that evidence, it is imperative that governments should take measures to screen oral cancer patients for HPV to improve survival and prognosis. In addition, immunization of under twenty-five against HPV should be encouraged as evidenced by the present research. HPV 16 is a major contributor to Head and Neck cancers. UK levels of vaccination for the target group in most regions according to the Public Health Observatory, is above 90%. Moreover, based on the results of the present study it is recommended that both sexes should be vaccinated as at present the vaccine is only administered to young girls.

The presence of HPV in HNSCC has been cited by some studies as a possible cause of better outcome for HNSCC patients (Lee *et al*. 2012; Duray *et al*, 2012). HPV-positive HNSCCs, according to Rautava *et al*. (2012), generally respond to radiotherapy and chemotherapy in comparison to HPV negative HNSCC and have a favourable disease-specific outcome. Although the exact mechanism is not fully understood, three possible explanations have been suggested (Rautava *et al*., 2012). The first suggested mechanism is that the genome of HPV-positive cancer cells is less unstable. The second mechanism is that HPV-positive cells suffer from hypoxia and can be more easily induced to apoptosis and the final mechanism is that treatment improves local immunity favouring the eradication of HPV and regression of the tumour.

The suggested better prognosis for HPV related in comparison to HPV unrelated HNSCC patients has been explained by some authors using a theory

that there is chemo radiation treatment induced the reduction in E6/E7 apoptosis induction in HPV positive tumours due to increased sensitivity to genotoxic treatments of HPV infected carcinomas (Na *et al*., 2007). However, according to Spanos *et al*. (2009) an immune reaction occurs important in the clearance of HPV positive tumours in addition to the direct cell toxic effects.

It has been suggested that the poor prognosis could be due to the biological action that HPV may produce in infected cells, which could stimulate the lymphocyte production of growth factors in the pharyngeal tongue and increase the cellular replication of HPV-infected cells (Kozomara *et al.*, 2005). Despite that improved prognosis, some HPV-positive patients still recur and HPV related HNSCC patients have a comparative rate of distant metastases to patients negative for HPV (Fakhry *et al*., 2008). Tobacco and smoking seems to worsen the disease outcome for all HNSCCs.

6.2 Tobacco, alcohol and HPV in HNSCC

The use of tobacco, was fuelled by industrialisation, improved transportation and mass media witnessed in the first half of the 20th century (Giovino *et al*., 1994). Tobacco use increased dramatically during the First World War and almost doubled in the Second World War due to economic expansion and prosperity in the industrialised world. The provision of cigarettes to troops during the World War Two was the foundation for the social use of tobacco by male users in the 20th century (Sturgis and Cinciripini, 2007). Dr Ernst Wynder 1950's research was the first to establish a link between cigarette smoking, lung cancer

and cancer of the mouth in separate studies carried out in 1950 and 1957 (Wynder and Graham, 1950; Wynder *et al*., 1957). A 5-fold increased risk between smoking and HNSCC was reported by further research in the UK and USA (Doll and Hill, 1956; Hammond and Horn, 1954).

Since the 1960's tobacco has been an established risk factor for HNSCC. Recent laws on smoking and government support on reducing the number of smokers as well as social acceptability of smoking behaviour was successful in reducing the number of smokers in the developed worlds (Alamar and Glantz, 2006). Though successful measures for tobacco control have been established, reductions in HNSCC incidences are not consistent for all sites across the head and neck regions especially the oral regions.

The present research reported 25.2% HPV positive biopsies with most of the biopsies from the tongue (61%) and mouth (12%) regions of the head and neck sites. Furthermore, most of the HPV positive biopsies (88.9%) were obtained from non-smokers. It therefore evident from the present research that the increase in HPV positive biopsies was not consistent with the tobacco laws that were enforced to reduce the number of smokers. There was no association established in the present research between smoking and HPV positivity, however an association was present between risky sexual behaviour and HPV positivity and SHC attendance ($p<0.05$).

Multiple studies have reported increased oral tongue cancers among adults (Depue, *et al*., 1986; Shemen, *et al*., 1984; Myers, *et al*., 2002). The study by

Byers (1975), was one of the first studies to report that phenomenon on HNSCC patients below the age of 30. The rises in HNSCC in the young adults have been linked by some research to the acquisition of the HPV in the affected individuals (Allison and Maleki, 2015; Fakhry *et al*., 2008). Sturgis and Cinciripini (2007) reported the observed inconsistency as being attributed to HPV genotype 16. Young adults presenting with HNSCC are mainly non-smokers with unknown history of alcohol abuse. Studies carried out in America on the presence of HPV in HNSCC biopsies have reported HPV positive HNSCC to be common in non-smokers compared to smokers (Allison and Maleki, 2016; Fakhry *et al*., 2008). Moreover, HPV positive HNSCC patients tended to be younger, non-smokers, non-alcoholics and of higher socioeconomic status.

The results from the present study showed most patients to be below 56 years of age. In addition, more than 88.9% had no history of smoking or alcohol abuse, that observation is consistent with research carried elsewhere (Allison and Maleki, 2016; Fakhry *et al*., 2008). In the present study, there was no female smoker and 10.4% of men were smokers with only 2 of these males testing positive for HPV. The smoking trends for participants in the present research showed older participants above the age of 60 to have a smoking history, with most of the non-smoking participants in the 51-60 age groups. It is of interest to note that most of the observed HPV positive participants were also in that age category. The above results suggest a relationship between smoking

history, age and HPV positivity, observations confirmed by obtaining statistically significant data ($p<0.001$).

Alcohol is another known risk factor for HNSCC, though some researchers argue that high rates of HNSCC is due to smoking with heavy alcohol consumption (Pandeya *et al*., 2013; Ogden, 2005). Alcohol consumption and smoking differ vastly between men and women. The present study reported all alcoholics to be of male gender with 3 in the 40-60 age groups and two above the age of 60. Results from the present study showed all HPV positive participants to be non-alcoholic males and females. Furthermore, statistical data showed non-alcoholics more likely to be HPV positive in comparison to alcoholics ($p<0.001$). Pandeya *et al*. (2013) in their study population estimated the fraction of alcohol consumption in relation to HNSCC, and reported a significant difference between men and women with 35% men and 5% women HNSCC's respectively attributed to alcohol and smoking combined. In the present research, there were a total of four cases (4% of participants) with a history of alcohol abuse and all of them were males; 50% of those males having a history of alcohol and smoking abuse combined. Furthermore, the 4% alcoholics that tested negative for HPV were all above the age of 60.

The results from the present study are in line with other research studies that reported reduced incidence of HPV positive HNSCC among alcoholics and smokers (Allison and Maleki, 2016; Fakhry *et al*., 2008). Contrarily, research on HPV and HNSCC on 222 specimens in Australia reported a very low prevalence

of HPV (3.6%), which leads to a conclusion that HPV is not associated with HNSCC (Antonsson *et al*., 2010). Interestingly, immunohistochemistry that is deemed a less sensitive method for detection in comparison to PCR, was incorporated in their research. The lack of connection between alcohol consumption and smoking with HPV positive HNSCC has prompted some researchers to examine other risk factors such as risky sexual behaviour (Farsi *et al*., 2015; Heck *et al*., 2010).

6.3 Racial distribution and HPV results

The aetiology of HNSCC over the past decades changed dramatically because of the steady decline in tobacco and alcohol related HNSCC but with increasing incidence of HPV related HNSCC (Young *et al*., 2015). Differentiation between HPV positive from HPV negative patients is now possible due to established patient demographic patterns. HPV positive HNSCC patients were described as relatively younger adults in their 40s and 50s non-smokers or with limited tobacco exposure. Recent evidence suggests HPV related head and neck cancers present with different symptoms than those caused by tobacco, with HPV HNSCC common in Caucasians of a higher socioeconomic status.

In the present study, 66% of the participants were males of Caucasian race with 32% of the participants, i.e. all females being of the Caucasian race. There was one male of African descent and two participants of Asian descent in the present research. The present research reported all HPV positive cases from individuals of Caucasian race as observed in other research studies where HPV

was associated with Caucasian race especially white males (Osazuwa-Peters, 2013; Young *et al*., 2015). Investigation of socioeconomic patterns was beyond the scope of this study. However, a study by Young *et al*. (2015) highlighted a link between HPV positive and socioeconomic status especially young adults in their 40s and 50s.

Better prognosis and survival rate have been associated with HPV positive HNSCCs in comparison to HPV negative HNSCCs. Zakeri *et al.* (2014) research on race and competing mortality in advanced HNSCC revealed that individuals of African descent were mainly associated with increased rates of comorbidity, smoking, heavy alcohol use, advanced tumour stage, and poorer performance status ($p < 0.001$ for all). The present research had only one participant of African descent and all HPV positive biopsies were obtained from Caucasians therefore, it was not possible to establish that association.

Other research studies reported association between HPV negative HNSCC (common in other races than Caucasians) with poor prognosis and survival rate (Mahal *et al*., 2014; Benson *et al*., 2014; Dahlstrom *et al*., 2015). Noticeable differences in survival rates between races may be in part or entirely explained by this difference in cause, HPV positive HNSCC are prevalent in Caucasians of male gender below the age of 55 as observed in the present research. Historically, HPV positive tumours of the HNSCC show better prognosis compared to HPV HNSCC. The increased survival rates in Caucasian could be pinpointed to the increased likelihood of HPV positive tumours in the Caucasian

race compared to other races. Most HPV positive biopsies tested in the present research were obtained from Caucasians.

6.4 HPV results and Sexual Health Clinic attendance

According to World Report (2013) just over 1.26 million of the UK population, yearly, attend SHCs in the UK. The risk of contracting sexually transmitted diseases is higher for individuals attending the SHC, however, the prevalence of sexually transmitted diseases remains unknown for the population not attending the clinics. SHC attendance was cited by Pista *et al*. (2012) as a risk factor for HPV including any reports for other sexually transmitted diseases.

A study reported a linear relationship between HNSCC and risk factors such as oral sex, multiple sex partners, and early age at first sexual encounter (Farsi *et al*., 2015). Lima *et al*. (2014) study on HIV positive and negative patients attending the sexual health clinic showed HPV infected individuals to be six times more likely to harbour oral and cervical HPV. HPV is the most widely recognized sexually transmitted disease (Dunne and Park, 2013). Data from the present UK study on 99 participants tested for HPV showed a strong correlation between SHC attendance and the risk of acquiring HPV ($p<0.001$), which is consistent with research in other countries that have linked sexually risky behaviour to the acquisition of HPV (Pista *et al*., 2012).

Cervical cancer, which is also caused by HR-HPV genotypes, has a similar aetiology with regards to the factors that lead to development of cancer. Sexually risky behaviour to include multiple sexual partners has been reported

to be one of the risk factors for cervical cancer. There have been reports of correlations between sexually transmitted infections and abnormal cytology as well as a sexually transmitted diseases and HR-HPV genotypes in the cervix. A similar pattern has been reported for HPV prevalence in the oral regions with a direct association between the number of sexually transmitted diseases and oral HPV prevalence in the UK population (Louie *et al*., 2015). Moreover, data collected for the present study showed high numbers of lifetime sexual partners and engaging in multiple sexual practices to be likely associated with visiting the SHC (UK NATSAL, 2010-2012). A recent report on rates of sexually transmitted infections in the UK revealed an upward trend with one fifth of the UK population reporting to have attended SHCs in the past five years; unsafe sexual behaviour was attributed to the noticed rise (Hughes and Lowndes, 2014).

Immunosuppressed patients are more prone to infections compared to individuals with a healthy immune system. Research carried out by Kriek *et al*. (2016) and Mourad *et al.* (2015) on the presence of HPV in biopsies from immunosuppressed HIV patients compared to HIV negative patients, showed HIV negative patients to have a reduced risk of acquiring HPV. The present research had only a single participant (1%) that was immunosuppressed; the results confirmed HPV carriage in this immunosuppressed participant. It is therefore possible that there is possible association between immunosuppression and acquisition of HPV in HNSCC. However, because there was only one HIV positive individual encountered in the present study, it is

difficult to draw conclusions on HPV prevalence in immunosuppressed HNSCC patients.

6.5 Natural history for HPV infection - Oral HPV infection and persistence in patients with HNSSCC

The natural history of HPV infection was extensively studied for the uterine cervix; however, there is less data available on the different phases of HPV infection and oncogenesis in the head and neck regions (Doorbar *et al.*, 2012). HPV persistence in the cervical mucosa for females, remains the strongest risk factor for invasive SCC and high-grade intraepithelial dysplasia and is associated with increased chances of tumour recurrence (Ramos *et al*., 2012). Cervical HPV infection and persistence can be regularly monitored on cervical exfoliated cells, which are useful for the molecular typing and identification of HPV (Dal Bello *et al.*, 2009).

Oral exfoliated cells have been suggested as a possible method that could be used to monitor infection with HPV in the oral cavity as well as risk of oropharyngeal SCC (Fakhry *et al*., 2011). D'Souza *et al*. (2009) study showed that infection with HPV is more typical in oral mucosa cells of patients with HNSCC contrasted with controls. To date scarce information is available on the persistence of viral infection after tumour treatment as well as its correlation with tumour recurrence (Chuang, *et al*., 2008).

The present research did not investigate the persistence of HPV in the oral mucosa of participants. The focus was on testing for the presence of HPV in

stored tissue samples on biopsy samples from HNSCC patients. As HPV persistence was not routinely tested for at Northampton hospital it is beyond the scope of the present research to comment on prior oral HPV persistence in patients who develop HPV positive HNSCC. However, research data to date suggest persistence of the oral HPV as a risk factor for HNSCC though more research is required on that subject (D'Souza *et al*., 2009). As highlighted by Fakhry *et al*. (2011) use of oral exfoliated cells to monitor HPV persistence should not be discounted.

Complications arose in defining the role of HPV infection in HNSCC due to various analytical methods available for virus detection, which use different targets and have different sensitivities and specificities (Morbini *et al*., 2013). Widely used PCR when compared with the gold standard of viral oncogene transcription, PCR based methods are not sufficiently specific, while p16INK4A (p16) immunostain and *in situ* hybridization (ISH) have low sensitivity (Westra, 2012). Determination of p16 expression and ISH are nonetheless currently the methods of choice in most laboratories to identify HPV-associated OSCC but cannot be easily applied to oral cytology monitoring, where HPV DNA amplification is the most convenient method to assess the presence of the virus.

Morbini *et al*. (2013) demonstrated that with PCR and reverse hybridization-based assays the presence of over 90% HPV genotypes in biopsies and oral mucosa samples, with an excellent agreement for HPV infection and genotype characterization between cancer and cytological samples. Their study

investigated the persistence of HPV infections in the oral region for patients with HPV related and HPV unrelated HNSCC after cancer treatment by means of repeated oral mucosa scrapings during patient follow up, to understand the natural history of HPV infection for the upper digestive tract and its correlation with disease recurrence and survival. PCR methodology, an easy, reliable and efficient method for detection of HPV was used for the present study. PCR methodology could be useful as a tool for assessing HPV persistence although more research is still required in that area.

6.6 The Biology and Life-Cycle of HPV

The life cycle of the papilloma virus and different types of epithelial disease caused by HPV (i.e. chronic asymptomatic infection or transient visible papillomas) has been linked to HPV's different strategies of transmission and propagation within the epithelium, and probably also to their different interactions with the immune system (Doorbar, 2005). HPV adapted to specific epithelial niches during evolution, with different types of HPV associated with different disease and disease prevalence (Gottschling *et al*., 2007; Shah *et al*., 2010). According to zur Hausen (2009) and Doorbar (2006) the most well studied HPV types are the mucosal Alpha types that cause cervical cancer and for these the biology of disease is relatively well understood.

The present study showed (92%) of the infections to be caused by HPV genotype 16. It is therefore possible that the mucosal alpha types causing disease in the cervix are now responsible for causing disease in the mucosal

sites of the head and neck sites as noted by the presence of primarily genotype 16 in the present study. HPV 16 life-cycle organisation at other important epithelial sites e.g. anus, penis, endocervix, penis and oropharynx is still poorly understood (Syrjänen *et al*., 2011; Wikstrom *et al*., 2012; Silva *et al*., 2011).

6.7 Prevention of HPV-driven cancer

HPV-associated cancer can be prevented firstly by minimising infection through HPV vaccination, and secondly through screening and interruption of disease. The vaccination route seems more promising as there is an HPV vaccine already on the market that has demonstrated high vaccine efficacy against one-time detection of oral HPV16/ 18 infection (Kreimer, 2014). Furthermore, indirect protection that entails reduction of HPV in the genital region should also reduce oral HPV exposure at the individual level.

Results from the present study also showed HPV vaccination to be above 90% in most regions of the UK. If the vaccine is deemed effective against oral HPV infection, the number of HPV HNSCC will be reduced in the future. At present, there are no validated screening methods for non-cervical HPV associated cancers. Serum HPV16 E6 antibody data suggest a possible future method to identify patients at risk prior to tumour development (Bouvard *et al*., 2009). Furthermore, presently biomarkers proven reliable, will require further research prior to use in clinical practice (Bouvard *et al*., 2009; Ferlay *et al*., 2010; de Martel *et al*., 2012).

Only one study, by Herrero *et al*. (2003), evaluated the prevention potential of the HPV vaccine against oral HPV infections. The vaccine trial study undertaken in Costa Rica evaluated vaccine efficacy against oral HPV infection in a randomized clinical trial (RCT) initially designed to evaluate vaccine efficacy against persistent cervical HPV16/18 infections and precancerous lesions. Mounting evidence that HPV was an entity in oropharyngeal cancers resulted in that aspect of the study being introduced four years following initial vaccination therefore, no pre-vaccination oral specimens were collected before the trial. HPV presence was only tested for once in that and efficacy of vaccine was estimated in some intention-to-treat participants. This included woman vaccinated regardless of baseline cervical HPV DNA or serology results, treatment for cervical pre-cancer or number of vaccine doses. Vaccine efficacy against HPV 16/18 of 93% was shown among women attending the study with one recorded infection in the vaccinated group in comparison to 15 in the control group. Vaccine efficacy was not analysed in the present study, that subject requires more research in the UK and worldwide.

According to Gillison *et al*. (2010) and Kreimer *et al.* (2011), guidelines recommend vaccination of females and males between the ages of 11 and 12 years. However, a study of HPV infection showed HPV prevalence to remain stable in early years and to increase with age (Kreimer, 2013). A research confirmed that acquisition at older ages (Kreimer *et al*., 2014). Therefore, for the vaccine to be effective it is imperative for it to last at least two decades after initial administration. Published data report protection of a maximum of eight

years, though there is no reassurance that protection will last that long (Roteli-Martins *et al*., 2012). A booster vaccine is a possibility if the vaccine is found to be effective against oral cancer, but more research is still required.

Future research designed to evaluate the efficacy of the HPV vaccine against oral HPV16/18 would need to address the collection of a pre-vaccine oral specimen to remove from the analysis individuals infected with oral HPV at the time of vaccination. In addition, future research should analyse persistent oral HPV16/18 rather than one-time detection of oral HPV infection, since the quantitative relationship between one-time detection of HPV in oral exfoliated cells and risk of future HPV-driven HNSCC is unknown. Lastly, considering of oral HPV infection and its likelihood to clear within one year, more large trials are required to help understand HPV persistence.

6.8 Obstacles for screening for HPV-driven oral cancer

Various obstacles remain at present and will need to be overcome before introduction of screening test for an HPV driven oral cancer. First, according to Kreimer, (2014) for the case of HPV-driven oral cancer, the question remains whether there is an identifiable precancerous lesion akin to the intra-epithelial lesions as at other HPV-driven cancer sites. Compared to head and neck cancers resultant from tobacco and alcohol use (and not HPV infection), there are the established precursors of leukoplakia and erythroplakia. Logically, HPV-driven cancers in the oropharynx in the head and neck regions do not erupt *de*

novo and it can be assumed there must be a continuum of detectable cancerous growth before invasive cancer.

No studies to date have effectively described this lesion, presence of HPV16 E6 antibodies 10 years before diagnosis with cancer, gives a possibility of the presence of precursor lesion or early cancer. Second, accessibility to the HPV head and neck cancer sites at which HPV causes cancer in the head such as base of tongue, and other parts of the pharynx, is difficult compared to anogenital sites for conventional diagnostic measures such as exploratory surgery including diagnostic biopsy. Thirdly, with oral cancer treatments available, survival rate is still below five years, therefore, new less invasive treatments are required. Fourthly, large-scale random controlled trials are still required to show that early detection of HPV driven HNSCC reduce mortality form the disease and confirmation that the benefits of screening outweigh the risks and costs. Furthermore, screening for HPV driven oral cancer can be hampered by the stigmatization that some authors have highlighted (Petti, 2009; Osazuwa-Peters *et al*., 2015). Finally, because HPV driven oral cancer is thought to be linked to sexual habits especially engaging in oral sex, depending on the culture, country, region of the world and individual beliefs and perspectives some individuals will be deterred from screening for oral HPV and vaccination programmes.

In the best of scenarios, because HPV-driven oral cancer is a fraction of head and neck cancer overall, even if all the HPV-driven HNSCCs were prevented

there would remain a burden of tobacco and alcohol -related head and neck cancers. The focus should also be reduction of tobacco and alcohol consumption. Substantial numbers of government backed programmes worldwide, including the USA and UK that have illegalised smoking in public.

6.9 Detection of HPV-DNA by a PCR-based Method

Extraction techniques for nucleic acids have a huge impact on the reliability of results obtained from molecular assays (Yang *et al*., 2011; Knepp *et al*., 2003). The extraction method for the present research - QIASymphony (S-392-n: QIAGEN, UK) - has been reported to have similar viral detection rates with other extraction platforms: easyMAG, bioMerieux; m2000sp, Abbott; MagNA Pure LC 2.0, Roche (Verheyena *et al*., 2012). Automated extraction systems provided for decreased hands-on time per sample and have vastly improved assay performance.

In other research, the results demonstrated that the QIAsymphony Sample Preparation and Assay Setup modules were highly reproducible and achieve equal performance, quantitative and qualitative, when compared with the NucliSens easyMAG and CAS-1200 systems for the molecular screening of enteric pathogens (van Zanten *et al*., 2011; Sahiner *et al*., 2014). Additionally, the QIAsymphony and QIAamp extraction platforms provided similar EBV DNA load values using whole blood (Laus *et al*., 2011). Results from the present study showed 23(92%) out of 25 biopsies to be positive for genotype 16, one biopsy tested positive for genotype 33 and the other biopsy failed to genotype.

HPV genotype 16 and 33 are categorised as high-risk oncogenic HPV subtypes. Oncogenic HPV 16 is responsible for most cervical cancer cases in the UK and worldwide. Oncogenic HPV 33 has been identified as a cause of cervical cancer in some countries but with a lower prevalence than HPV 16. The assay tested for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 that are considered carcinogenic. The majority (92%) of the samples tested were positive for HPV 16 and 4% positive for genotype 33.

A recent study reported that HPV 16 in precancerous and cancerous lesions promote malignant phenotypes by influencing cancer stem cell populations by incorporation of miRNA mediated epigenetic regulation which further dictates progression to cancer (Sung *et al*., 2015). This study provided evidence that high-risk HPV16 enhances malignant growth and cancer stem cell phenotype of HPV-negative oropharyngeal squamous carcinoma cells by suppressing tumour suppressive miRNAs, miR-181a and miR-181d. In that study, the researchers investigated the role of HPV16 in promoting the virulence of HPV negative ORCC cells. The investigation was carried out by introducing HPV16 whole genome in HPV-negative oropharyngeal squamous cells. The results showed increased malignant growth and self-renewal capacity, a key characteristic of cancer stem cells. In a review by Allison and Maleki (2016), the overall HPV prevalence in HNSCC is reported to be 26%. A significant HPV prevalence in oropharyngeal (35%) was reported in the same review in comparison to oral squamous cell carcinoma (23.5%) was reported (Allison and Maleki, 2016).

The estimate confirms results obtained in the present study, which showed a 25.2% prevalence of HPV positive HNSCC. Alison and Maleki (2016) also highlighted that HPV 16 is more commonly detected in oropharyngeal SCCs (86.7%) compared to laryngeal SCCs (69.2%) and oral SCCs (68.2%). The notion was supported by another review which highlighted HPV 16 as the most common cause of oral squamous cell carcinomas (Boscolo-Rizzo *et al*., 2016). Conversely, HPV 18 is less commonly detected in HPV-related oropharyngeal SCCs (2.8%) (Allison and Maleki, 2016). The results are consistent with results obtained in the present study where HPV 16 accounted for all positive biopsies except one which was identified as genotype 33 and another that could not be identified to genotype level.

Chapter 7: Oral Cancer Incidence Worldwide

The present study investigated trends in incidence of HNSCC worldwide by sex, age in all the subsites of oral cancer (nasopharynx, lip, oral cavity, larynx, oesophagus and pharynx). The recent data from GLOBOCAN (2012) indicate an overall increase in HNSCC incidences for males and females in all age groups. According to Joseph and D'Souza, (2012) there are more than 600,000 incident cases of HNC worldwide each year, with the majority from the oral cavity. The incidence of oral cavity, larynx, and pharynx cancer varies widely by geographic region and gender (Chaturvedi *et al*., 2008). In all age groups and sites the number of men affected is higher in comparison to females. The highest numbers of oral cancer incidences were seen in Europe (16.2%) and Asian (64.6%), in comparison to Africa (5.9%) and the Oceanic (0.9%). Research has suggested that the differences seen in the continents in the number of oral cancer are associated with different risk factors (Joseph and D'Souza, 2012; Chaturvedi *et al*., 2008).

The present research analysed differences in incidences between developed and less developed countries. The incidence rates for HNSCC were higher in less developed countries (mainly Asian) in comparison to more developed countries. The different risk factors for positive HPV HNSCC and non-HPV HNSCC has led to the noted differences in incidences in around the world. HNSCC in the Asian subcontinent is mainly due to use of tobacco in that region, especially betel quid. The noted differences around the globe could be due to the differential exposure to the risk factors for HNSCC. Though tobacco and

smoking still pose a risk factor for acquiring HNSCC, worldwide tobacco laws in most developed countries have substantially reduced the number of smokers.

Tobacco use has been declining among both men and women, although its use remains more prevalent among men (Sturgis and Cinciripini, 2007). Data from PHE 2015 from the present research showed smoking to be declining in the UK especially among males, though males generally have a higher trend of smoking compared to females. The higher smoking trend in males offers a possible explanation for increased incidence of HPV unrelated HNSCC in males. Reasons for the increased incidence of HPV HNSCC have not yet been established. Increased sexual behaviour, which consequently increases exposure to oral sex, has consistently been associated with increased odds of developing HPV positive HNSCC (Joseph and D'Souza, 2012).

The oral cancer incidence is on the rise despite the tobacco laws that have substantially reduced the number of smokers in developed countries. The 5-year survival rate for HNSCC is currently (61%) and (48%) for females and males respectively (Hertrampf *et al*., 2015). The biopsies tested for HPV in the present research had 25.2% positivity. The rise of oral cancer incidences has been attributed to HPV positive HNSCC with 25% of all HNSCC in developed worlds currently caused by HPV (Hertrampf *et al*., 2015; Van Dijk *et al*., 2012).

7.1 Oral cancer incidence and mortality in Europe

Data was extrapolated from the WHO and GLOBOCAN databases on overview of cancer incidence estimate in Europe from 1975 to 2010. The data showed

HNSCC cancer incidences across Europe to be on the rise for both males and females. Several research have reported the noted trend in oral cancer incidences. Ferlay *et al*. (2015) recently published data on oral cancer incidences in Europe, in which there was a general increase of the number of oral cancers across the European subcontinent; supported by the present research. Currently, oral cancer now the seventh most common cancer is responsible for 16.2% of the all new cases yearly in Europe (IRAC/WHO, 2014). According to Van Dijk *et al*. (2012) 24,000 HNSCC cases are diagnosed yearly in Europe. Hertrampf *et al*. (2015) reported an estimated of 100,000 new oral cancer cases yearly in Europe and 64.6% new cases in the Asian Subcontinent. The higher number of oral cancer incidence in the Asian subcontinent is attributed to the widespread use of betel quid. The African subcontinent reported a very low cancer incidences of 5.9%.

Risk factors for oral cancer are age, male gender, tobacco and alcohol and HPV (Krüger *et al*., 2014). The European subcontinent's incidence of ORCC and oral cavity cancers has varied greatly by country and gender, with some countries experiencing higher increases in the age standardized incidence rates over the last decade. The UK, Denmark, Slovakia, Spain, France and Finland are among European countries reporting increased numbers of oral cancer incidences in both sexes. GLOBOCAN data from the present study on oral cancer incidences in those countries showed a slow increase of oral cancer from 1975-2011 with peak increases noted between the years of 2005 to 2010 in both sexes. There has been an increase of tongue carcinoma noted in some countries including

the United States (Depue, 1986; Karim-Kos *et al*., 2008). These increases were reported in those under 55 years of age mainly in the 30–50-year age groups. Changes in sexual behaviour, leading to increased oral HPV infection, is deemed likely to contribute to the increased incidence of oral carcinoma in those below 60.

7.2 Oral Cancer Incidence in the UK

The UK oral cancer incidence rates were comparable to the increases that were reported in European. Cancer Research UK data from the year 1975 to 2011 showed increased cancer incidences in both sexes. The years 1975-1977 and 2009-2011 showed increases of 202% in males and 131% in females. Louie *et al*. (2015) reported similar trends in oral cancer incidences for the UK. Louie *et al*. (2015) research also reported high incidences of HNSCC paralleling with increased STI diagnoses (proxy for HPV infection) for males and females in the UK, showing a change in sexual behaviour for both sexes in recent years. Oropharyngeal carcinoma incidences were reported to have risen in parallel to the incidence of genital warts and genital herpes diagnosis between 1975 and 2011 (Louie *et al*., 2015).

Data from Cancer Research UK on UK oral cancer incidences in males and females showed a general upward trend for both sexes. However, in males there has been a substantial reduction in oral cancer incidences in those above 80 years old, with the oral cancer incidence reduced by almost 50% over the period 1975 to 2011; 62 and 33 per 100,000 respectively. The noticeable

increases in oral carcinoma has been attributed to the large increases observed in the relatively younger age groups mainly the 50-59 year olds. The results from the present research parallel that observation, where a mean age of 56.2 was observed for both sexes with males having a mean age of 55.5. Data from Cancer Research on oral cancer incidences according to age showed incidences in males to be high in the 45-64-year age groups and then a sharp decline after the age of 65. The peak age of diagnosis for females was even lower at 40-44 years of age. The results from the present study showed the mean age for females to be 57.8. Only 32 female participants were analysed in the present study therefore results obtained may not reflect the incidences in the general population.

The present research data on peak age of diagnosis compared to data from Cancer research is different perhaps due to limited numbers of research participants. Cancer Research UK data however, highlight the observed trend in the present research of HNSCC in young females. The observed lower age of peak diagnosis for female (40-44 years) from Cancer research database which was lower than the age reported for the present research (57.8) showing the rise of HNSCC in females in the UK. Cancer research UK data, also showed a rise in number of younger males diagnosed with HNSCC. Other research studies have reported a high number of oral cancer incidences in males below the age of 60 (Chaturvedi *et al*., 2013; Louie *et al*., 2015). Deschler *et al*. (2014) suggested that the rise in oral cancer in the younger age groups could be attributed to HPV. A study by Louie *et al*. (2015) showed 25% of all HNSCC to

be attributed to HPV driven HNSCC. The results from Louie *et al*. study was supported by other studies undertaken elsewhere (Young *et al*., 2015; Heck *et al*., 2010). The present study, where 25.2% of the biopsies tested positive for HPV supports the results reported above by the other research.

There has not been much research about HPV and HNSCC in the UK, and research in the USA (Chaturvedi *et al*., 2008; Gillison *et al*., 2012), and Canada (Pintos *et al*., 2008) and Greece (Blioumi *et al*., 2014) have reported similar trends. Other research has estimated that 70-80% of new oropharyngeal cancer diagnosed to be HPV driven (Deschler *et al*., 2014; Young *et al*., 2015). This percentage is far higher than that observed in the present research (25.2%). The observed differences could be attributed to the type of samples analysed; the present research tested mainly tongue and mouth biopsies whilst the above research studies results were based on oropharyngeal biopsies.

To investigate whether trends in the UK on oral cancer were parallel to the East Midlands region where this research was carried out, data on oral cancer incidences in the East Midlands was extrapolated from PHE-London Knowledge and Intelligence Team (based on data extracted from National Cancer Registration Service's cancer registration system ENCORE). The East Midlands region of the UK contributed a total of 1,687 of the 18,936-total number of oral cancer incidences in the UK. The Northampton region had the fourth highest number of cancer incidences in the region (244); with Derbyshire (311) having the highest number followed by Nottinghamshire (308) and Lincolnshire (255).

Therefore, from the researcher's point of view the Northampton region of the UK could closely reflect the general trend of cancer incidences in the UK. A research study in the UK by Louie *et al*. (2015) examined oral cancer incidences by subsite. Results from their study for the years 1995 to 2011 showed a rise of 58.9% in HNSCC cases (Louie *et al*., 2015). In their research the highest increase was greatest for the base of tongue, in comparison to other sites. The present research reviewed data from Cancer Research UK, and obtained similar results where the highest numbers of reported oral cancer cases in 2010 were from the tongue region (1,336). The results were also consistent with results from biopsies tested for HPV where 61% of cases were from the tongue region.

The probable potential source of bias in the present study is the use of different cancer registries to extrapolate data on cancer incidences worldwide. Cancer registration is dynamic and, therefore, there might be differential reporting across regions because of the different resources and infrastructures established to support each registry. However, despite these concerns, because the results show a higher incidence of oral cancer in the northern regions such as Scotland where the resources for capturing cases might have been more scarce, it implies that the Cancer Research' findings that oral cancer is rising across the United Kingdom is sound and maybe even understated. The Cancer Research' data also revealed a differential increase in oral cancer incidence rates in younger age groups. That observation was confirmed in the

present study where (61%) of HPV positive biopsies were identified in the tongue regions of those below the age of 56.

Chapter 8: Sexual Behaviour

8.1 Changes in sexual behaviour in the UK

The UK in comparison to other countries worldwide has witnessed a significant change in sexual behaviour in the past three decades. Data from the present study showed significant changes of behaviour in the UK population in sexual behaviours such as age at first sexual experience, total number of lifetime partners, engagement in oral, anal and vaginal sex. High-risk sexual behaviour such as an increased number of lifetime partners has always been linked to an increased likelihood of acquiring sexually transmitted diseases. These sexually risky behaviours consequently lead to the acquisition of sexually transmitted diseases which eventually results in individuals attending SHC for treatment. HPV is a well-known sexually transmitted disease and a study by Farsi *et al*. (2015) reported a strong correlation between HPV incidences and SHC attendance.

Limited studies on oral HPV and sexual behaviour have been carried out in the UK. Recent studies of sexual behaviour and HPV were carried out in the USA, India, Poland, Italy and Cuba (Beachler and D'Souza, 2010; D'Souza *et al*., 2009; Garrote *et al*., 2001; Gillison *et al.,* 2008; Lissowska *et al*., 2013;). All these previous studies observed elevated risks of high numbers of sexual partners with increased risk of acquisition of HPV related HNSCC with different rates between men and women. Consistent estimates were observed by two studies that examined sexual behaviours only among positive HPV patients

(Gillison *et al*., 2008; Garrote *et al*., 2001). Comparison of risk factors of HPV related and HPV unrelated cancers was carried out in a study by Gilllison *et al*. (2010) and strong associations between sexual behaviours and HNSCC were reported among HPV positive persons only in comparison to HPV negative patients. Furthermore, the studies grouped all head and neck sites together in their investigations and did not subdivide them into their sub-sites. The present research reported HPV positivity in the different subsites of the head and neck sites with most of the HPV positives being reported in the tongue and mouth regions (61% and 12% respectively).

The largest study carried out on the association between sexual behaviours and HPV was by Farsi *et al*. (2015). They reported that one of the leading risk factors for HPV acquisition as a high number of lifetime sexual partners. The present study investigated UK sexual behaviour trends in the past three decades. There was an observed trend towards having more lifetime partners in the younger generations compared to the older generations. NATSAL survey (1990-1991) reported male average number of 8.6 partners, the number gradually rose with the reported average number of sex partners rising to 12.6 in NATSAL survey (1999-2011) and 11.7 in NATSAL's (2010-2012) survey. Similar increases were noted for women, with only 3.7 average number of sex partners NATSAL (1990-1991), that number further increased to 6.5 (NATSAL 1999-2001) and 7.7 (NATSAL 2010-2012).

A study by Farsi showed elevated point estimates and cancer of the base of the tongue as linked to two lifetime sexual partners; at least four lifetime oral sex partners were strongly associated with a 3-fold increase in tonsil cancer risk. In addition, Farsi *et al*. (2015) reported an increased risk of base of the tongue and oropharyngeal cancer with individuals having two sexual partners compared to one. Baseman and Koutsky, (2005) reported a similar observation. Sexual behaviour studies often report men as having more sexual partners than women. However, Cook *et al*. (2014) argue that those reports are due to male exaggeration and female underreporting.

The present research obtained data on archival biopsies on whether there was a history of attending the SHC. SHC attendance was significantly associated with the likelihood of testing positive for HPV ($p<0.05$). This is in line with another UK study that reported a link between the percentages of the average sexually transmitted infections with the percentage of oral HPV infections (Louie *et al*., 2015). Cook *et al*. (2014) however, found no connection between sexually transmitted infection and HPV prevalence.

Age at first sexual encounter has been associated with risky sexual behavior to include multiple sexual partners and inconsistent protective as well as increased alcohol and smoking (Gillison *et al*., 2010; Farsi *et al*., 2015). Age at first sexual encounter can therefore, be used as a marker for possible riskier sexual behavior often leading to acquisition of HPV positive HNSCC. Data from the present study showed age at first sexual encounter to be dropping consistently,

the reported age of first sexual experience for those born in 1935 was eighteen, data for those born in 1996 showed that age had dropped to at least 15.5 or less for males in the UK. It could be inferred that the observed trend has continued to 2016 with the age of first sexual encounter reducing continuously, however there is no available data to support that.

A similar trend was observed in the present study for females with the age of first intercourse in for those born in 1935 reported to be 19.5 compared to those born in 1996 where the age of first intercourse for females was below 15.5. The present research also revealed more young people to be having sex before the age of 16 compared to the older counterparts. The 16-24-year old reported an average of 31 and 29 % for males and females respectively to have engaged in sex before the age of 16. In contrast, the 65-74-year old reported 15 and 4% of males and females respectively to have engaged in sex before the age of 16. The data from the present also indicated that the more the number of lifetime sexual partners the more the likelihood of visiting the SHC ($p<0.05$).

In studies undertaken in the USA, Australia, Russia and Brazil generational changes in sexual behavior have been reported, with the younger generations of individuals who came of age in recent years being reported as having more numbers of lifetime partners, an earlier age at sexual experience and an increased likelihood of engaging in oral sex in comparison to older generations (Chervyakov and Kon, 2000; Caron and Moskey, 2000; Comportamento, 2000; Haddow *et al*., 2006). That observation was confirmed in the present research,

which also showed an increased tendency among the younger generations to have more lifetime partners which consequently leads to visiting the SHC.

Database results from the present study therefore, showed a relationship between the number of sexual partners an individual reported to be associated with the likelihood of attending the SHC consequently leading to acquisition of HPV related HNSCC. The associations reported in the present study provide additional indirect evidence that HPV is an entity of HNSCC especially in the tongue and mouth cancers. However, more studies should be carried out to determine whether HPV vaccines can prevent HPV infection and ultimately HNSCC.

Most studies on sexual behavior and HPV to date were retrospective in nature. Questions used in most studies were like those used for studies on cervical cancer and sexual behaviour, thereby suggesting sufficient information was available to detect an association (Heck *et al*., 2010). One limitation is the validity of self-reported sexual behaviours (NATSAL survey data), with concerns for underreporting of stigmatized behaviours. Nonetheless, reporting in NASTAL surveys reveals that increased sexual partners and engaging in sex of any form (oral, anal, vaginal) as an increasing trend. Average age at first sexual encounter has significantly decreased, and published NATSAL data in this study reported higher numbers of individuals attending the SHC. However, studies to date have failed to investigate fully other potential risks for HNSCC

such as diet that has been mentioned by some sources (Cancer Research UK; Heck *et al*., 2008).

8.2 Oral/Anal/Vaginal sex

The UK data from UK NATSAL surveys showed a significant number of the UK population to be practicing sexually risky behaviour in recent years. The oral sex trends data from NATSAL reported in the present study showed an increased number of younger age groups in both sexes to have engaged in oral sex. Oral sex was reported as one of the major risk factor for HNSCC (Heck *et al*., 2010; Farsi *et al*., 2015). Persistent oral HPV carriage sometimes leads to development of HPV positive HNSCC. As noted in the present study, data from NATSAL surveys showed oral sex percentage increased as age decreased for those that reported to having practiced oral sex. PCR biopsy analysis for HPV from the present study, also revealed most of the HPV positive biopsies to be from individuals below the age of 56. A study by van Monsjou *et al*. (2013) on HPV and squamous cell carcinoma in the head and neck regions on younger patients reported a disproportionate increase of the HNSCC in those below the age of 45. Though that group only constituted 5% of all HNSCC, the distinct phenotype of HNSCC in that age group was attributed to HPV exposure.

Younger age and oral sex, as well as age at first sex encounter and multiple partners are associated with HPV HNSCC. People that engage in oral sex were reported to have a tendency of engaging in other sexual practices and are more likely have multiple partners (Cook *et al.,* 2014; Farsi *et al.,* 2015). According to

Van Monsjou *et al*. (2013) HPV positive HNSCC are characterised by low rates of self-reported alcohol consumption and smoking, and increased rates of self-reported promiscuity and marijuana use. In a study, different kinds of sex e.g. oral, anal and casual sex and inconsistent condom use showed statistically significant association with risk for HNSCC (Tachezy *et al*., 2009). That observation was confirmed by other studies that showed high numbers of lifetime oral sex partners (>5) as well as vaginal sex partners (>25) to be linked with high risk of HPV related HNSCC (Tay and Oon, 2014; D'Souza *et al*., 2014).

Results from the present study were in line with the above research' showing participants attending the SHC to be at higher risk of acquiring HPV. Furthermore, data from the present research also showed patients attending the SHC to be individuals with risky sexual behaviours to include a higher number of sexual partners and likely to have engaged in other sexual practices such as oral and anal sex. NATSAL data on sexual trends from the present study revealed a significant number of the UK population increasingly engaging in sexually risky behaviour which could explain the noted increase incidence of HNSCC in the UK especially HPV positive HNSCC possibly being transmitted via the sexual route.

Kissing with regards to the number of partners of open-mouth kissing has been reported to have an association with oral HPV infection, and would consequently be a pertinent exposure to consider. To date, open mouth kissing

and HNSCC have not been thoroughly investigated however, kissing and transmission of HPV was beyond the scope of the present study. Koskimaa *et al*. (2011) indicated the possibility of vertical transmission; persistent oral in infection in mothers was associated with persistent HPV oral infection in their infants, suggesting a non-sexual route of transmission.

There is still very little or no evidence for auto-inoculation or other non-sexual transmission, although it is difficult to exclude these possibilities. It has been suggested that that infections could be acquired orally and genitally from the same infected partner during oral and vaginal sex. To date that seems to be the most likely route of transmission for oral HPV though more research is required to investigate possibility of HPV genital–oral transmission in the same individual. The present study did not investigate oral and genital HPV status of participants as the study mainly focused on the presence of HPV in biopsies of patients with HNSCC.

In another study, a low correlation for oral and cervical HPV infections within the same individual was reported (Fakhry *et al*., 2006). Statistical investigations in some studies on cervical and oral HPV infection as well as anal and oral HPV infection showed no significant associations between oral HPV infection and HPV infection in the cervix or anal regions (Parisi *et al.*, 2011; Palefsky *et al*., 2001). According to Chung *et al*. (2014), data from the above studies strongly suggest HPV oral infections are sexually transmitted during oral sex on infected genitals or by rimming an infected anus. Risk factors associated with oral HPV

HNSCC infection from previous studies on oral HPV incidences have been identified as male gender, tobacco exposure and risky sexual behaviours (Gillison *et al*., 2009; Kreimer *et al*., 2011).

A study by Gillison *et al.* (2012) reported oral HPV prevalence to be 8-fold higher among sexually active participants compared to non-sexually active participants and increased with number of lifetime or recent sexual partners for any kind of sex including oral and vaginal sex. In addition, that prevalence was highest amongst participants reporting more than 20 lifetime sexual partners. That data was supported by the present study that showed patients with risky sexual behaviour (as measured by attendance of the SHC) to be highly likely to be HPV positive compared to patients with low risk sexual behaviour. The data from the present study on sexual trends in the UK, was in line with the above research showing individuals with higher number of sexual partners to be highly associated with oral HPV positivity. According to the data from the present study, individuals with a higher number of sexual partners (more than 11 and 8 for males and females respectively) were more likely to engage in any kind of sex including oral, vaginal, anal and vaginal sex.

8.3 Limitations of study and future work

The present study carried in Northampton at a district hospital in the UK had limitations as to the number of individuals that could be tested for HPV due to availability of resources and time limits. Thus, 99 participants were included in the study. However enough data was generated to produce a statistically

significant result that were comparable to studies elsewhere. Further multiple small scale studies or large scale studies are deemed necessary in the UK to further study the burden posed by the noted increase in HNSCC and the risk factors associated with it.

The study was carried out on paraffin embedded tissue biopsies that had been stored from 2006 to 2014. It was therefore difficult to obtain data on clinical response to treatment and rates of survival for HPV positive and HPV negative patients. Clinical studies carried out from the point of diagnosis to at least 5 years after diagnosis are necessary in the UK to identify whether there is a difference in survival rate between positive HPV and negative HPV tumours. The study was limited to Northampton where most on the patients with positive biopsies were of white origin, it will be of interest to carry out a UK wide research where more patients from diverse backgrounds are included in the study to further analyse the racial disparity associated with HNSCC on a large scale.

8.4 Summary

At least 25% of all HNSCCs of the head and neck regions are thought to be HPV related. The incidence of HNSCC carcinomas has increased over the past 30 years in developed countries and have been attributed to HPV-related carcinomas. An epidemiologic study in London UK recently showed a HNSCCs rise in both sexes with significant variation by cancer site mainly in oropharyngeal and oral cancers (Tataru *et al*., 2016). Oral HPV infections are

sexually acquired and seem to be quickly cleared by most individuals but persist in a subset of infected individuals.

The role of HPV in causing oropharyngeal cancer is clearly established, but it is less clear what role HPV may play in other head and neck subsites. The first objective of the present study was the examination of the prevalence of HPV in biopsies from patients with HNSCC at Northampton Hospital. Limited research has been carried out in the UK in that area. The HPV prevalence rate of 25.2% from biopsies of the oral and tongue regions reported in the present study was comparable to results reported elsewhere other than the UK e.g. Brazil and USA (Sand and Jalouli, 2014; Syrjänen *et al*., 2011). Research elsewhere have attributed the noted increase of HPV HNSCC to be linked to sexual behaviour.

The present study examined that aspect of the sexual behaviour as a possible contributing factor to the rise of HPV positive HNSCC by collection of data from publicly available databases available in the UK. To examine whether sexual behaviour could influence HPV positivity in HNSCC the present study examined whether participants had attended the SHC. The data showed an association between sexual behaviour and HPV positivity and measured by attendance of the SHC ($p<0.05$). However, large scale studies are imperative to establish HPV and association with HNSCC especially in the UK where very few studies have been conducted in that area. Other research' have reported HPV related carcinomas to be prevalent in those below the age of 55 (Young *et al*., 2015; Allison and Maleki, 2016: Dahlstrom *et al*., 2015).

The present study used participants' data from clinical records on age of HNSCC patients and compared it statistically to HPV positivity. The results from the present study were in line with research elsewhere were all HPV positive biopsies were obtained from patients less than 55 years of age (Young *et al*., 2015). Furthermore, future studies that focus on the natural history of oral HPV infections could help to further understanding of HPV-HNSCC. Presently, the UK and worldwide limited research has been done in that area. As tobacco-related HNSCC continues to decrease, as noted in the present study, HPV may continue to emerge as an increasingly important cause of HNSCC. Researchers reported HPV HNSCC patients likely to be white, middle-aged (45-60), and to have had more oral sexual partners (Chu *et al*., 2016; Dahlstrom *et al.*, 2015).

Sexual behaviour, as data from the present study revealed, has changed vastly in the past 40 years in the UK. Data from UK NATSAL showed more people in the UK practicing sexually risky activity such as having multiple partners and engaging in any kind of sex including oral sex which has been identified as one of the highest risk causes of HPV related HNSCC (Gillison *et al*., 2009; Kreimer *et al*., 2011). It is therefore of prime importance that more research in carried on the prevalence of HPV in HNSCC in the UK and the possible impact of the HPV vaccine on HPV related HNSCC. More research is still required to establish whether immunization protects against HPV HNSCC or whether a booster is needed for adequate protection.

The clinical prognosis of HPV positive HNSCC compared to HPV HNSCC participants was beyond the scope of the present research. Clinically, HPV-positive HNSCC behaves differently with a better prognosis, but with a different pattern of distant metastases and fewer second primary malignancies (D'Souza *et al*., 2016). Studies need to figure out if treatment can be reduced without diminishing survival in HNSCC patients. Clinicians ought to know about the diverse epidemiologic variables and clinical behaviours related with HPV-positive HNSCC to efficiently diagnose and treat patients. Since most HPV-positive HNSCC cases are caused by HPV 16, as reported in the present study, immunization against HPV 16 amid adolescence and before HPV presentation may later reduce development of HPV-positive HNSCC.

Future research to investigate the adequacy of the quadrivalent vaccine (Gardasil) and bivalent vaccine (Cervarix) against HPV related HNSCC is still required. Besides, clinicians and research centres ought to accomplish more to separate between HPV related and HPV unrelated HNSCC through offering routine HPV testing to all possible HNSCC patients. Finally, primary prevention strategies should be aided by education programmes for dentists, coupled with routine examinations of the oral cavity to increase chances of early detection and treatment of patients (Hertrampf *et al*., 2013).

Appendices

Appendix 1: Extraction protocol

Extraction

For reproducibility purposes, all samples prepared as detailed in below are routinely extracted on the QIASymphony (S-392-n).

Proteinase K

Proteinase K is taken from the QiaSymphony extraction kit (Qiagen). Alternatively, QIAGEN Protease (Mat No. 1045135) maybe used in place of Proteinase K.

Reserve stocks of Proteinase K reconstituted according to the manufacturer's instructions (High Pure Viral Nucleic Acid Kit) are stored at -70ºC and maybe used for nucleic acid extraction using the High pure Viral Nucleic acid kit.

Buffer ATL

Please note, preparation of the ATL buffer may require gentle warming to dissolve precipitates that form during prolonged storage. For samples being extracted on the QIAsymphony with the exception of 16S and *E. coli*, buffer ATL (QIAGEN # 19076) should be supplemented with phage Φ-174

(30µL of a 10-2 dilution (located in fridge 3) per 50mL Buffer ATL) and proteinase K.

Phage-free buffer ATL supplemented with proteinase K ONLY should be used for samples being tested for 16S and *E. coli* to reduce contamination. DO NOT use AL buffer from the MDx robot for these samples since it contains phage Φ-174 propagated in *E. coli* (This poses a contamination risk to these assays).

PolyA

The poly A solution used is identical to that used on the MDX BioRobot and is stored in 50µL aliquots in freezer. Buffer should be supplemented with polyA (5µL per tube) for low cellular content samples (CSF, Vitreous samples, corneal swabs). PolyA should be added to the sample in the processing tube prior to the addition of the ATL/proteinase K.

Isopropanol

20µl of Isopropanol should be added to any samples, which appear to be 'bubbly' following the final incubation at 72°c and prior to extraction on the QiaSymphony. This is particularly the case for samples that have undergone treatment on the TissueLyser. Failure to treat such samples with Isopropanol may result in incorrect assessment of liquid levels contained in tubes by the carbon-based tips used by the QiaSymphony.

Paraffin wax embedded fixed tissue

Place 5-10 tissue sections (or scrapings from a block) into a labelled microcentrifuge tube and add 1ml xylene. Cap the tube and invert several times. Centrifuge at 13,000 rpm for 1 minute.

Carefully remove the supernatant without disturbing the tissue pellet and discard into the designated xylene discard bottle. Re-suspend the tissue in a further 1ml of xylene, centrifuge and discard the supernatant into the xylene discard bottle. If the supernatant is still opaque a further xylene wash is required.

Add 1ml of absolute ethanol to the tissue, centrifuge and remove the supernatant. Repeat the ethanol wash, carefully removing as much ethanol supernatant as possible with a pipette tip. Add 1ml Elution Buffer from the High Pure Viral Nucleic Acid Kit (transparent cap), centrifuge and discard the supernatant. Resuspend the sample thoroughly by vortexing. Add 180µl Buffer ATL and 20 µl proteinase K to the sample, vortex briefly and incubate at 55°C with shaking (500 oscillations per min) for 3-24 h until the sample appears homogeneous. ATL buffer may require gentle warming to dissolve precipitates that form during prolonged storage. During longer incubations (e.g. overnight) an additional 20 µl proteinase K should be added after the initial 3 hours. If the sample has not fully dissolved after an overnight incubation, use the TissueLyser with 5mm stainless steel bead as in 3bi above. Spin down briefly and add 40µL Proteinase K and 200µL of ATL Buffer to each sample. Incubate at 72°C on the heating block for 10min, spin down, and proceed to extraction on the QiaSymphony (S-392-n).

Appendix 2: PCR and Sequencing Methodology

PCR protocol

Reagents

5x PCR buffer (Labmaster)

Taq polymerase enzyme (Labmaster)

10mM dNTPs solution (Bioline)

Molecular grade water (Severn Biotech)

Oligonucleotide primers are routinely sourced from Sigma-Genosys in lyophilised form along with the manufacturer's datasheet. This states, amongst other details, the total yield in nMol. As a default, the lyophilised pellet is re-suspended in an appropriate volume of PCR grade water to give a 50µM master stock concentration.

Primers for HPV_O single target semi-nested PCR are diluted 1:10 from the master stocks to give 5µM working solutions.

Primers for HPV_N single target semi-nested PCR are diluted according to primer degeneracy and range from 0.375µM to 5.75µM.

Agarose (Bioline)

TBE (Invitrogen)

Ethidium Bromide (Sigma-Aldrich)

6x loading buffer (contains 0.25% bromophenol blue/Xylene cyanol, 60% glycerol, 40% water)

Sure-Clean (Bioline)

Big Dye version 3 Reaction ready mix (ABI)

ABI sequencing buffer (ABI)

Glycogen Solution (Roche)

EDTA (Sigma-Aldrich)

NaOAc Solution (Sigma-Aldrich)

Absolute Ethanol (95% minimum) (Fluker)

HiDi Formamide (ABI)

Special equipment

Both HPV target assays use a semi-nested PCR. Amplification is performed on a standard thermocycler, and detection is via gel electrophoresis.

UV Transilluminator

If a positive is found via gel electrophoresis, then the product is sequenced on an ABI 3130xl genetic analyser.

Preparation

Mix preparation (see table below) should be performed in the clean room ONLY and template addition in the general laboratory area or template transfer hoods.

Reagent	First round quantity (µL)	Second round quantity (µL)
5x Buffer	5	5
dNTPs	5	5
Primer	1	0.5
Water	20	40
Taq polymerase	1	1
PCR aliquot per reaction	30	48

i) First round reactions

20µL of extracted nucleic acid is added to first round mixes

ii) Second round reactions

2.4µL of the first reaction mix is transferred to the respective second reaction in the template transfer cabinet using a filter pipette tip and multi-channel pipette.

iii) Internal controls

All assays run should include the appropriate number of positive and negative controls. In-use positive control material for PCR is kept in fridge at 4°C or freezer at -70°C.

PCR Program

First Round

Initial Denaturation: 2 minutes at 95°C

Denaturation: 30 seconds at 95°C

Annealing: 30 seconds at 50°C

Extension: 30 seconds at 72°C

Repeat for 40 cycles

Second Round

Initial Denaturation: 2 minutes at 95°C

Denaturation: 30 seconds at 95°C

Annealing: 30 seconds at 40°C

Extension: 30 seconds at 72°C

Repeat for 30 cycles

Gel Electrophoresis

Agarose (9g) was mixed with 0.5X TBE (300mL) and the solution heated until fully boiling. Ethidium bromide (15µL) was added and once cooled poured into a tray with a comb inserted and left to set.

Once set the comb is removed, and the gel placed into a gel tank. This was covered by 0.5X TBE to a depth of 2mm.

Load 10µL of each sample with 6X Loading Buffer into the slots created by the comb insert.

Connect the tank to the power pack and run at 250V (Current limit: 250mA, Power limit 25W) for 23 minutes.

Once finished, check gel on UV transilluminator

Sequencing

Purify PCR products (40µl) using 'Sure-Clean'. Add an equal volume (40µl) of 'Sure-Clean' reagent to the PCR product, vortex briefly to mix and follow by 5-10 minutes incubation at room temperature. Centrifuge at full speed (13,000 RPM) for 5-10 minutes (Note: All the tubes should be orientated in the same direction with the hinge facing up. This is so that the position of the pellet, which may not be visible, can be predicted), then carefully remove all of the supernatant (it may be necessary to perform another pulse spin and then aspirate using a fine tip e.g. P10.). Re-suspend in pre-warmed distilled water, varying the final re-suspension volume in accordance with the PCR reaction

yield. Use up to 50µl for faint bands 100µl for typical intensity bands and 150-200µl for very bright bands.

Add the following to a suitably labelled 0.2 mL PCR tube and mix by gentle pipetting.

4µl of Molecular Biology grade water

2µl of purified/diluted PCR product (template)

2µl of Big Dye version 3 Reaction ready mix

1µl of ABI sequencing buffer

1µl of sequencing primer (5µM)

Place reaction tubes on the thermal cycler, and run a PCR program with the parameters below:

- Denaturation: 30 seconds at 96°C
- Annealing: 15 seconds at 50°C
- Extension: 2 minutes at 60°C

Repeat for 25 cycles

Prepare the required amount of ‘stop solution’ and mix by vortexing. The volumes below are sufficient for 16 samples.

18µl Glycogen Solution

36µl 0.1M EDTA Solution

36µl 3mM NaOAc Solution (pH 5.2)

180µl Molecular Biology grade water

Add 15µl of stop solution and 60µl of cold (-20°C) absolute (95% acceptable) ethanol directly to each 10µl sequencing reaction. Mix by vortexing briefly, and centrifuge at maximum speed (13,000 RPM) for 15 minutes.

Carefully aspirate the supernatant, taking care not to disturb the region around the pellet.

Add 200µl of cold (-20°C) 70% ethanol and vortex briefly. Centrifuge again at maximum speed for 10 minutes.

Carefully aspirate the supernatant. Note: at this stage, it is vital to remove ALL the liquid, so it will be necessary to perform another pulse spin and then aspirate using a fine tip (e.g. P10).

Re-suspend the pellet in 12.5µl of HiDi Formamide by vigorous vortexing.

Finally, transfer 10µl of the resuspensions (using an 8-channel pipette) to the next available row of an ABI sample loading plate.

Place the ABI sample plate in the PCR block and heat the samples for 2 minutes at 95°C and then freeze for 1 minute at -20°C, before placing onto the sequencer.

Appendix 3: Sex of Participant, Smoking history, Age Groups

Age Groups			Sex of Participant		Total
			Male	Female	
Age 20-60	Smoking history	Smoker	6	2	8
		Non-Smoker	40	16	56
	Total		46	18	64
Age 60-70	Smoking history	Smoker	0	2	2
		Non-Smoker	14	9	23
	Total		14	11	25
Age above 70	Smoking history	Smoker	1	0	1
		Non-Smoker	6	3	9
	Total		7	3	10
Total	Smoking history	Smoker	7	4	11
		Non-Smoker	60	28	88
	Total		67	32	99

Appendix 4: HPV positivity and alcohol use in relation to sex

Sex of Participant			HPV result				Total
			HPV 16 positive	HPV negative	HPV 33 positive	HPV positive unknown genotype	
Male	Drinking history	alcoholic	0	4		0	4
		Non-alcoholic	18	44		1	63
	Total		18	48		1	67
Female	Drinking history	Non-alcoholic	5	26	1		32
	Total		5	26	1		32
Total	Drinking history	alcoholic	0	4	0	0	4
		Non-alcoholic	23	70	1	1	95
	Total		23	74	1	1	99

Appendix 5: Estimated Incidence and prevalence of head and neck cancer in the adult population in both sexes in Europe GLOBOCAN 2012

Larynx, Lip, oral cavity, Oesophagus, Other pharynx, Nasopharynx - Estimated incidence and prevalence, adult population: both sexes					
POPULATION	*Quality	Incidence	1-year (prop.)	3-year (prop.)	5-year (prop.)
Hungary	G1	4282	2897 (34.1)	6573 (77.4)	8929 (105.2)
Belgium	A2	3567	2638 (29.4)	6381 (71.2)	9016 (100.6)
Germany	B2	26894	19467 (27.4)	48676 (68.5)	71025 (99.9)
Croatia	A2	1309	994 (26.6)	2451 (65.6)	3523 (94.2)
France (metropolitan)	B2	18986	14360 (27.7)	34120 (65.9)	47465 (91.7)
Portugal	C3	3513	2471 (27.1)	5877 (64.5)	8250 (90.6)
Serbia	B2	2510	1978 (24.3)	4893 (60.2)	7039 (86.5)
The Netherlands	A2	4884	3323 (24.1)	8031 (58.1)	11449 (82.9)
Denmark	A2	1619	1107 (24.1)	2618 (56.9)	3663 (79.6)
Switzerland	B2	2045	1517 (23.1)	3633 (55.3)	5076 (77.2)

Spain	B2	11238	8205 (20.7)	20516 (51.8)	29950 (75.6)
Luxembourg	D2	115	90 (20.8)	216 (50.0)	308 (71.3)
Romania	E1	6107	4056 (22.4)	9380 (51.7)	12926 (71.3)
Montenegro	G6	127	97 (18.9)	250 (48.7)	364 (71.0)
Malta	A1	97	70 (19.5)	176 (49.1)	253 (70.5)
United Kingdom	A1	18483	11010 (21.2)	25769 (49.7)	36230 (69.8)
Slovenia	A1	509	361 (20.6)	860 (49.0)	1223 (69.8)
Czech Republic	A2	2632	1852 (20.4)	4355 (48.1)	6056 (66.9)
Poland	C3	9166	6368 (19.5)	15155 (46.4)	21186 (64.9)
Austria	A2	1889	1350 (18.7)	3235 (44.8)	4573 (63.3)
Italy	B2	11682	8874 (16.9)	22547 (43.1)	33018 (63.1)
Slovakia	A1	1552	992 (21.3)	2148 (46.1)	2822 (60.5)
Republic of Moldova	G1	787	556 (18.9)	1280 (43.6)	1755 (59.8)
Bulgaria	A2	1710	1196 (18.8)	2755 (43.3)	3781 (59.5)
Bosnia	D5	630	496 (15.5)	1252 (39.1)	1839 (57.4)

Herzegovina					
Belarus	A2	2236	1457 (18.0)	3274 (40.5)	4432 (54.9)
Iceland	A1	52	38 (14.6)	96 (36.9)	142 (54.5)
Ireland	A1	987	584 (16.3)	1381 (38.4)	1945 (54.1)
FYR Macedonia	G3	269	224 (13.1)	615 (35.8)	925 (53.9)
Finland	A1	972	683 (15.1)	1681 (37.3)	2429 (53.8)
Ukraine	A2	10145	6671 (17.4)	15089 (39.3)	20455 (53.3)
Norway	A2	885	616 (15.2)	1495 (37.0)	2144 (53.0)
Sweden	A2	1616	1143 (14.4)	2828 (35.7)	4091 (51.7)
Lithuania	A1	763	477 (17.0)	1072 (38.2)	1450 (51.7)
Latvia	A1	500	310 (16.2)	699 (36.5)	946 (49.4)
Russian Federation	D2	28427	17766 (14.7)	39875 (33.1)	53901 (44.7)
Estonia	A1	253	165 (14.6)	369 (32.7)	501 (44.4)
Albania	G3	392	291 (11.5)	739 (29.2)	1083 (42.8)
Greece	G3	1314	972 (10.0)	2452 (25.2)	3570 (36.7)

Cyprus	A3	74	56 (6.0)	143 (15.3)	207 (22.1)
POPULATION	*Quality	Incidence	1-year (prop.)	3-year (prop.)	5-year (prop.)
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Slovakia	A1	1552	992 (21.3)	2148 (46.1)	2822 (60.5)
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References

Ajab, H., Yasmeen, S., Yaqub, A., Ajab, Z., Junaid, M., Siddique, M. *et al.* (2008). Evaluation of trace metals in tobacco of local and imported cigarette brands used in Pakistan by spectrophotometer through microwave digestion. *Journal of Toxicological Science* 33(4): 415–420.

Alamar, B. and Glantz, S. A. (2006) Effect of increased social unacceptability of cigarette smoking on reduction in cigarette consumption. *American Journal of Public Health* 96: 1359–1363.

Albero, A., Villa, L. L., Lazcano-Ponce, E., Fulp, W., Papenfuss, M. R., Nyitray, A. G., *et al. (*2013) Male circumcision and prevalence of genital human papillomavirus infection in men: a multinational *study*, *Biomed Central Infectious Diseases* 13:18

Albers, A., Abe, K., Hunt, J., Wang, J., Lopez-Albaitero, A., Schaefer, C. *et al*. (2005) Antitumor activity of human papillomavirus type 16 E7-specific T cells against virally infected squamous cell carcinoma of the head and neck. *Cancer Research* 65: 11146–55.

Allison, D. B. and Maleck, Z. (2016) HPV-related Head and Neck Squamous Cell Carcinoma: An Update and Review Article. *Journal of the American Society of Cytopathology* 1-13.

Ang, K. K., Harris, J., Wheeler, R., Weber, R., Rosenthal, D. I., Nguyen-Tan, P. F. *et al*. (2010) Human papillomavirus and survival of patients with oropharyngeal cancer. *New England Journal of Medicine* 363(1): 24–35

Annertz K, Anderson, H., Palmér, K. and Wennerberg, J. (2012) The increase in incidence of cancer of the tongue in the Nordic countries continues into the twenty-first century. *Acta Otolaryngologica*, 132(5): 552-7.

Antonsson, A., Nancarrow, D. J., Brown, I. S., Green, A. C., Drew, P. A., Watson, D. I. *et al.* (2010) High-risk human papillomavirus in esophageal squamous cell carcinoma. *Cancer Epidemiology Biomarkers Preview* 19(8): 2080–7.

Arbyn, M. and Dillner, J. (2007) Review of current knowledge on HPV vaccination: An Appendix to the European Guidelines for Quality Assurance in Cervical Cancer Screening. *Journal of Clinical Virology* 38: 189-197.

Arnson, Y., Shoenfeld, Y., Amital, H. (2010) Effects of tobacco smoke on immunity, inflammation and autoimmunity. *Journal of Autoimmunity* 34: 258–65.

Arulkumaran, S., Regan, L., Papageorghiou, A., Monga, A. and Farquharson, D. (2011). *Oxford Desk Reference: Obstetrics and Gynaecology* Oxford University Press pg. 471.

Attner, P., Du, J., Nasman, A., Hammarstedt, L., Ramqvist, T., Lindholm, J., *et al*. (2011) Human papillomavirus and survival in patients with base of tongue cancer. *International Journal of Cancer* 128(12): 2892–7

Axford, S. E., Ogden, G. R., Stewart, A. M., Saleh, H. A., Ross, P. E. and Hopwood, D. (1999). Fluid phase endocytosis within buccal mucosal cells of alcohol misusers. *Oral Oncology* 35: 86–92.

Bahl, A., Kumar, P., Dar, L., Mohanti, B. K., Sharma, A., Thakar, A., *et al*. (2013) Prevalence and trends of human papillomavirus in oropharyngeal cancer in a predominantly north Indian population, *Head and Neck* 6: 1

Baseman, J. G. and Koutsky, L.A. (2005) The epidemiology of human papillomavirus infections, *Journal of Clinical Virology* 32(1): S16–S24

Baumann, J. L., Cohen, S., Evjen, A.N., Law, J. H., Vadivelu, S. and Attia, A. *et al*. (2009) Human papillomavirus in early laryngeal carcinoma *Laryngoscope* 119(8): 1531–7.

Beachler, D. C and D'Souza, G. (2010) Nuances in the changing epidemiology of head and neck cancer. *Oncology* 24(10): 924–6.

Beachler, D. C., Abraham, A. G., Silverberg, M. J., Jing, Y., Fakhry, C., Gill, M. J. *et al.* (2014) (On behalf of the North American AIDS Cohort Collaboration on Research and Design (NA-ACCORD) of IeDEA) Incidence and risk factors of HPV-related and HPV-unrelated Head and Neck Squamous Cell Carcinoma in HIV-infected individuals *Oral Oncology* 50: 1169–1176

Beachler, D. C., D'Souza, G., Sugar,E.A., Xiao, W. and Gillison, M. L. (2013) Natural History of Anal vs Oral HPV Infection in HIV-Infected Men and Women. *Journal of Infectious Diseases* 208(2): 330-339.

Beachler, D. C., Weber, K. M., Margolick, J. B., Strickler, H. D., Cranston, R. D., Burk, R.D *et al.* (2012) Risk factors for oral HPV infection among a high prevalence population of HIV positive and at-risk HIV-negative adults. *Cancer Epidemiology Biomarker Prevention* 21: 122–33 (A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology).

Beachler, D.C. and D'souza, G. (2013) Oral human papillomavirus infection and head and neck cancers in HIV-infected individuals. *Current Opinion in Oncology* 25(5): 503–10.

Begum, S., Cao, D., Gillison, M., Zahurak, M. and Westra, W. H. (2005) Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. *Clinical Cancer Research* 11(16): 5694–9.

Begum, S., Gillison, M. L., Nicol, T. L. and Westra, W. H. (2007) Detection of human papillomavirus-16 in fine-needle aspirates to determine tumour origin in patients with metastatic squamous cell carcinoma of the head and neck. *Clinical Cancer Research* 13(4): 1186–91.

Benson, E., Li, R. Eisele, D. and Fakhry, C. (2014) The clinical impact of HPV tumour status upon head and neck squamous cell carcinomas. *Oral oncology* 50: 565–574.

Berkowitz, Z., Malone, M., Rodriguez, J. and Saraiya, M. (2015) Providers' beliefs about the effectiveness of the HPV vaccine in preventing cancer and their recommended age groups for vaccination: Findings from a provider survey, 2012. *Preventive Medicine* 81: 405–411.

Bhattacharya, A., Roy, R., Snijders, A. M., Hamilton, G., Paquette, J., Tokuyasu, T. *et al*. (2011) Two Distinct Routes to Oral Cancer Differing in Genome Instability and Risk for Cervical Node Metastasis. *Human Cancer Biology* 17: 7024.

Bishop, J. A., Ogawa, T. and Chang, X. (2012) HPV analysis in distinguishing second primary tumours from lung metastases in patients with head and neck squamous cell carcinoma. *American Journal of Surgical Pathology* 36: 142-148

Blioumi, E., Chatzidimitriou, D., Pantzartzi, C. H., Katopodi, T. H., Tzimagiorgis, G., Emmanouil-Nikoloussi, E. N., *et al*. (2014) Detection and typing of Human

Papillomaviruses (HPV) in malignant, dysplastic, nondysplastic and normal oral epithelium by nested Polymerase Chain Reaction, immunohistochemistry and transitional electron microscopy in patients of Northern Greece *Oral Oncology* 50(9): 840-847

Blot, W. J., McLaughlin, J. K., Winn, D. M., Austin, D. F., Greenberg, R. S., Preston-Martin, S. *et al*. (1988) Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Research* 48: 3282–3287.

Boscolo-Rizzo, P., Pawlita, M. and Holzinger, D. (2016) From HPV-positive towards HPV-driven oropharyngeal squamous cell carcinomas *Cancer Treatment Reviews* 42: 24–29

Bray, F., Ren, J. S., Masuyer, E. and Ferlay, J. (2008) Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *International Journal for Cancer* 132(5): 1133-45

Bryan J. T., Taddeo F., Skulsky D., Jansen K. U., Frain B. M., Qadadri B., *et al.* **(**2006**)** Detection of specific human papillomavirus types in paraffin-embedded sections of cervical carcinomas. *Journal of Medical Virology* 78: 117–124

Byers, R. M. (1975) Squamous cell carcinoma of the oral tongue in patients less than thirty years of age. *American Journal of Surg*ery 130: 475–478.

Byrd, J. K., Wilhoit, C. S., Fordham, M. T., Reeves, T. D., McRackan, T. R., Nguyen, S. A., *et al*. (2012) Predicting HPV status in head and neck cancer: the predictive value of sociodemographic and disease characteristics. *Archives Otolaryngiology Head Neck Surgery* 138: 1155–59.

Caberg, J. H., Hubert, P., Herman, L., Herfs, M., Roncarati, P. and Boniver, J. *et al.* (2009) Increased migration of Langerhans cells in response to HPV16 E6 and E7 oncogene silencing: Role of CCL20. *Cancer Immunol Immunother* 58: 39–47.

Cancer Research UK (2015) Cancer Statistics [Online] Cancer Research UK Available from http://info.cancerresearchuk.org/cancerstats/faqs/#How Prepared by Cancer Research UK - Available from http://www.cancerresearchuk.org/cancer info/cancerstats [Accessed 05.08.15]

Cancer Research UK (2015) Oral Cancer Statistics [Online] Cancer Research UK. Available from: http://www.cancerresearchuk.org/about-cancer/type/mouth-cancer/?gclid=CMTYrv-2-MUCFQXLtAod-1YAEA&dclid=CL3uvv-2 MUCFQpYfAodscsAJQ [Accessed 01.05.15]

Cancer Research UK (2015) Oral Cancer Statistics [Online] Cancer Research UK. Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/oral-cancer/incidence#heading-One [Accessed 01.02.16].

Cancer Statistics Registrations England (2012) [Online] Cancer Statistics Registrations England. Available from http://www.ons.gov.uk/ons/rel/vsob1/cancer-statistics-registrations--england--series-mb1-/index.html [Accessed 01.08.15]

Carlson, E. R. (2016) HPV Associated Oral and Oropharyngeal Cancer *Journal of Oral and Maxillofacial Surgery* 74(4): 682-683)

Caron, S. L. and Moskey, E. G. (2002) Changes over time in teenage sexual relationships: comparing the high school class of 1950, 1975, and 2000. *Adolescence* 37: 515–26.

Carpenter, L. M. (2009) A Tale of Two Technologies HPV Vaccination, Male Circumcision, and Sexual Health *Gender & Society* 23(6): 790-816

Chaturvedi A. K, Engels E. A, Pfeiffer R. M, Hernandez B. Y, Xiao W, Kim E, *et al*. (2011) Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of Clinical Oncology* 29(32): 4294–301

Chaturvedi, A. K., Engels, E. A., Anderson, W. F., Gillison, M. L. (2008) Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. *Journal of Clinical Oncology* 26(4): 612–9.

Chervyakov, V. and Kon, I. (2000) Sexual revolution in Russia and the tasks of sex education. In: Moatti JP, Souteyrand Y, Prieur A, Sandfort T, Aggleton P, editors. AIDS in Europe*: New Challenges for the Social Sciences*. Routledge, London.

Chu, K. P., Habbousa, P., Kuanga, Q., Boyda, K., Mirshamsa, M., Liub, F. F., *et al*. (2016) Socioeconomic status, human papillomavirus, and overall survival in head and neck squamous cell carcinomas in Toronto, Canada *Cancer Epidemiology The International Journal of Cancer Epidemiology, Detection, and Prevention* 40: 102–112

Chung, C. H., Bagheri, A. and D'Souza, G. (2014) Epidemiology of oral human papillomavirus infection *Oral Oncology* 50: 364–369

Cnattingius, S., Galanti, R., Grafstrom, R., Hergens, M.P., Nyren, O., Pershagen, G. (Eds.), (2005). *Health Risks from Swedish Snuff. Karolinska institute,* pp. 1–95.

Cogliano, V., Baan, R., Straif, K., Grosse, Y., Secretan, B. E. l. and Ghissassi, F. (2005) Carcinogenicity of Human papillomavirus *Lancet Oncology* 6: 204

Cohen, M. A., Basha, S. R., Reichenbach, D. K., Robertson, E., Sewell, D. A. (2008) Increased viral load correlates with improved survival in HPV-16-associated tonsil carcinoma patients. *Acta Otolaryngologica* 128(5): 583–9.

Colditz, G.A. and Dart, H. (2016) Epidemiology and Associations Between Diet and Cancer *Cancer; Reference Module in Biomedical Sciences* Update of Encyclopedia of Human Nutrition (Third Edition) (2013) pp 247-252

Collins, S., Upshaw, J., Rutchik, S., Ohannessian, C., Ortenberg, J. and Albertsen, P. (2002) Effects of circumcision on male sexual function: debunking a myth? *Journal of Urology 167*: 2111-2112

Conway, D. I., Stockton, D. L., Warnakulasuriya, K. A., Ogden, G., Macpherson, L. M. (2006) Incidence of Oral and Oropharyngeal Cancer in United Kingdom (1990-1999)—Recent Trends and Regional Variation *Oral Oncology* 42(6): 586-92

Cook, M. B., McGlynn, K. A., Devesa, S. S., Freedman, N. D. and Anderson, W. F. (2011) Sex Disparities in Cancer Mortality and Survival *Cancer Epidemiological Biomarkers Preview* 20(8): 1629–37.

Cook, R. L., Thompson, E. L., Kelso, N. E., Friary, J., Hosford, J., Barkley, P. *et al*., (2014) Sexual behaviors and other risk factors for oral human papillomavirus infections in young women, *Sexually Transmitted Dis*eases 41(8): 486–492

Coughlan, D., Keogh, I. and O'Connor, T. (2013) HPV and Head and Neck Cancer *Cancer Professional* 7: 3

Curado, M. P., Edwards, B., Shin. H. R., Storm. H., Ferlay. J., Heanue. M., *et al.* (2007) Cancer Incidence in Five Continents, Vol. IX. *IARC Scientific Publications* No. 160, Lyon, IARC.

D'Souza, G. and Dempsey, A. (2011) The role of HPV in head and neck cancer and review of the HPV vaccine *Preventive Medicine* 53: S5–S11

D'Souza, G., Anantharaman, D., Gheit, T., Abedi-Ardekani, B., Beachler, D. C., Conway, D. I. *et al*. (2016) Effect of HPV on head and neck cancer patient survival, by region and tumour site: A comparison of 1362 cases across three continents *Oral Oncology* (62): 20-27

D'Souza, G., Kreimer, A. R, Viscidi, R., Pawlita, M., Fakhry, C., Koch, W. M. *et al.* (2007) Casecontrol study of human papillomavirus and oropharyngeal cancer *New England Journal of Medicine* 356(19): 1944–56.

D'Souza, G., Zhang, H. H., D'Souza, W. D., Meyer, R. R., Gillison, M. L. (2010) Moderate predictive value of demographic and behavioural characteristics for a diagnosis of HPV16-positive and HPV-16negative head and neck cancer. *Oral Oncol*ogy 46: 100–4

D'Souza, G., Agrawal, Y., J. H., Bodison, S., and Gillison, M. L. (2009) Oral sexual behaviours associated with prevalent oral human papillomavirus (HPV) infection. J*ournal of Infectious Disesase* 199: 1–7.

D'Souza, G., Fakhry, C., Sugar, E. A., Seaberg, C. C., Weber, K., Minkoff, H. L., *et al*., (2007). Six-month natural history of oral versus cervical human papillomavirus infection. *International Journal of Cancer* 121(1): 143–50.

Dahlstrom, K. R., Bell, D., Hanby, D., Li, G., Wang, L. E., Wei, O., *et al.* (2015) Socioeconomic characteristics of patients with oropharyngeal carcinoma according to tumour HPV status, patient smoking status, and sexual *Oral Behaviour Oncology* 519: 832-838

Deocaris, C. C., Taira, K., Kaul, S. C and Wadhwa, R. (2005) Mimotope-hormesis and mortalin/grp75/mthsp70: a new hypothesis on how infectious disease-associated epitope mimicry may explain low cancer burden in developing nations *FEBS Letters* 579: 586-590

Depue, R. H. (1986) Rising mortality from cancer of the tongue in young white males. *New England Journal of Medicine* 315(10): 647.

Deschler, D. G., Richmon, J. D., Khariwala, S. S., Ferris, R. L. and Wang, M. B. (2014) The ''new'' head and neck cancer patient-young, nonsmoker, nondrinker, and HPV positive: evaluation. *Otolaryngology Head Neck Surgery* 151(3): 375–80.

Doll, R. and Hill, A. B. (1956) Lung cancer and other causes of death in relation to smoking. A second report on British doctors *British Medical Journal* 2: 1071–1081.

Du, X., Squier, C. A., Kremer, M. J. and Wertz, P. W. (2000). Penetration of N-nitrosonornicotine (NNN) across oral mucosa in the presence of ethanol and nicotine. *Journal of Oral Pathological Medicine* 29: 80–85.

Dunne, E. F. and Park, I. U. (2013) HPV and HPV-Associated Diseases. *Infectious Disease Clinics of North America* 27(4): 765–778

Eyre, H., Kahn, R., Robertson, R. M., Clark, N.G., Doyle, C., Gansler, T., *et al.* (2004) Preventing cancer, cardiovascular disease, and diabetes: a common agenda for the American Cancer Society, the American Diabetes Association, and the American Heart Association. *A Cancer Journal for Clinicians* 54: 190–207.

Fahey, L. M., Raff, A. B., Da Silva, D. M., Kast, W. M. (2009) A major role for the minor capsid protein of human papillomavirus type 16 in immune escape. *Journal of Immunology* 183: 6151–6

Fakhry, C. and D'Souza, G (2013) Discussing the diagnosis of HPV-OSCC: Common questions and answers *Oral Oncology* 49: 863–871.

Fakhry, C., D'souza, G., Sugar, E., Weber, K., Goshu, E., Minkoff, H. *et al*. (2006) Relationship between Prevalent Oral and Cervical Human Papillomavirus Infections in Human Immunodeficiency Virus-Positive and -Negative Women *Journal of Clinical Microbiology* 44(12): 4479–4485

Fakhry, C., Marksc, M. A., Gilmand, R. H., Cabrerrae, L., Yorid, P., Kosekd, M., *et al*. (2013) Comparison of the immune microenvironment of the oral cavity and cervix in healthy women *Cytokine* 64(2): 597–604.

Fakhry, C., Westra, W. H., Li, S., Cmelak, A., Ridge, J. A., Pinto, H. *et al*., (2008) Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *Journal of the National Cancer Institute* 100(4): 261–9.

Falk, R. T., Pickle, L. W., Brown, L. M., Mason, T. J., Buffler, P. A. and Fraumeni, J. F. (1989) Effect of smoking and alcohol consumption on laryngeal cancer risk in Coastal Texas. *Cancer Research*. 49: 4024 - 4029.

Farsi, N. J., El-Zein, M., Gaied, H., Lee, Y. C. A., Hashibe, M., Nicolau, B., *et al*. (2015) Sexual behaviours and head and neck cancer: A systematic review and meta-analysis *Cancer Epidemiology* 39: 1036–1046

Fenkl, E. A., Schochet, E., Jones, S. G. and da Costa, B. R. (2015) Evaluation of an HPV/Anal Cancer Screening Awareness Program for HIV-infected Men Who Have Sex With Men. *Journal of the Association of Nurses in AIDS Care* 26(4): 492-497

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers' C., Rebelo, M., *et al*. (2015) Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012 *International Journal of Cancer* 136(5): E359-E386

Fink, K. S., Carson, C. C. and DeVellis R. F. (2002) Adult circumcision outcomes study: effect on erectile function, penile sensitivity, sexual activity and satisfaction. *Journal of Urology* 167: 2113-2116

Fleiss, P. M. and Hodges, F. M. (2002) What Your Doctor May Not Tell You About Circumcision. *New York, NY: Warner Books*; 2002

Forman, D., Bray, F., Brewster, D. H., Gombe Mbalawa, C., Kohler, B., Piñeros, M. *et al.* (2013) *Cancer Incidence in Five Continents, Vol. X* [Online] Lyon, IARC Available from http://ci5.iarc.fr Accessed on [01-11-2013]

Fowles, J. and Dybing, B. (2003) Application of toxicological risk assessment principles to the chemical constituents of tobacco smoke. *Tobacco Control* 12: 424–430

Fox, N., Hunn A. and Mathers, N. (2009) The NIHR RDS for the East Midlands / Yorkshire & the Humber 2009 14: Sampling and Sample Size Calculation

Franceschi, S., Levi, F., La Vecchia, C., Conti, E., Maso, L. D., Barzan, L. *et al.* (1999) Comparison of the effect of smoking and alcohol drinking between oral and pharyngeal cancer. *International Journal of Cancer* 83: 1-4.

Franceschi, S., Talamini, R., Barra, S., Barón, A. E., Negri, E., Bidoli, E., *et al*. (1990) Smoking and drinking in relation to cancers of the oral cavity, pharynx, larynx, and oesophagus in northern Italy. *Cancer Research* 50: 6502 - 6507.

Frazer, I. H. and Levin, M. J. (2011) Paradigm shifting vaccines: prophylactic vaccines against latent varicella-zoster virus infection and against HPV associated cancer *Current Opinion in Virology* 1: 268-279

Friedrich, R. E. and Kristen, U. (2003) Toxicity assessment of mouthwashes in the pollen tube growth test. *Anticancer Research* 23: 941–947

Frisch, M., Smith, E., Grulich, A., Johansen, C. (2003) Cancer in a population-based cohort of men and women in registered homosexual partnerships. *American Journal of Epidemiol*ogy 157: 966–72

Gagari, E. and Kabani, S. (1995). Adverse effects of mouthwash use. A review. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontics* 80: 432–439

Garrote, L. F., Herrero, R and Reyes, R. (2001) Risk factors for cancer of the oral cavity and oro-pharynx in Cuba, Br. *Journal of Cancer* 85(1): 46–54.

Gillison M. L, Koch W. M, Capone R. B, Spafford M, Westra W. H, Wu L, *et al*. (2000) Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *Journal National Cancer Institute* 92(9): 709–20.

Gillison, M. L and Lowy, D. R. (2004) A causal role for human papillomavirus in head and neck cancer *Lancet* 363(9420): 1488–9.

Gillison, M. L, D'Souza, G., Westra, W., Sugar, E., Xiao, W., Begum, S., *et al*. (2008) Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16 negative head and neck cancers. *Journal National Cancer Institute* 100(6): 407–20

Gillison, M. L. (2009) HPV and prognosis for patients with oropharynx cancer. *European Journal of Cancer* 45(1): 383–5.

Gillison, M. L. Castellsague, X., Chaturvedi, A., Goodman, M.T., Snijders, P., Tommasino, M. *et al*. (2014) Eurogin roadmap: comparative epidemiology of HPV infection and associated cancers of the head and neck and cervix. *International Journal of Cancer* 134: 497–507.

Gillison, M. L., Broutian, T., Pickard, R. K. (2012) Prevalence of oral HPV infection in the United States, 2009–2010. *Journal of American Medical Association* 307: 693–703.

Gillison, M. L., Zhang, Q., Jordan, R., Xiao, W., Westra, W. H, Trotti, A., *et al*. (2012) Tobacco smoking and increased risk of death and progression for patients with p16-positive and p16-negative oropharyngeal cancer. *Journal of Clinical Oncol*ogy 30: 2102–11.

Giovino, G. A., Schooley, M. W., Zhu, B. P, Chrismon, J. H., Tomar, S. L., Peddicord, J. P. *et al*. (1994) Surveillance for selected tobacco-use behaviors—United States, 1900–1994. MMWR *Morbidity Mortality Weekly Report*. 43(SS-3): 1–43.

Giskes, K., Kunst, A. E., Benach, J., Borrell, C., Costa, G., Dahl, E., *et al.* (2005) Trends in smoking behaviour between 1985 and 2000 in nine European

countries by education. *Journal of Epidemiology and Community Health*. 59: 395–401.

Glick, L. B. (2005) Marked in your flesh: Circumcision from Ancient Judea to Modern America. *New York, NY: Oxford University Press*; 2005

GLOBOCAN (2012) Estimated Cancer Incidence, Mortality, Prevalence Worldwide 2012 [Online] GLOBOCAN. Available from http://globocan.iarc.fr. [Accessed 2.06.15]

Golas, S. M. (2007) Trends in palatine tonsillar cancer incidence and mortality rates in the United States. *Community Dental Oral Epidemiology* 35(2): 98-108.

Goncalves, P. H., Montezuma-Rusca, J. M., Yarchoan, R. and Uldrick, T. S. (2016) Uldrick Cancer prevention in HIV-infected populations *Seminars in Oncology 43(1): 173-188*

Habbous, S., Chu K, P., Qiu, X., Delfa, A. L., Harland L. T. G., Fadhel., *et al*. (2013) The changing incidence of human papillomavirus-associated oropharyngeal cancer using multiple imputation from 2000 to 2010 at a Comprehensive Cancer Centre *Cancer Epidemiology* 37: 820–829

Haddow, L. J., Dave, B., Mindel, A., McPhie, K. A., Chung, C. and Marks, C. *et al*. (2006) Increase in rates of herpes simplex virus type 1 as a cause of anogenital herpes in western Sydney, Australia, between 1979 and 2003. *Sexually Transmitted Infection* 82: 255–59.

Hammarstedt, L., Lindquist, D., Dahlstrand, H., Romanitan, M., Dahlgren, L. O., Joneberg, J. *et al*. (2006) Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer, *International Journal for Cancer* 119: 2620–2623

Hammond, E. C. and Horn, D. (1954) Relationship between human smoking habits and death rates: follow-up study of 187,766 men. *Journal of the American Medical Association* 55: 1316–1328.

Hashibe, M., Brennan. P, and Chuang, S. C. (2009) Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer Epidemiology Biomarkers Preview* 18(2): 541–50.

Heath, S., Willis, V., Allan, K., Purdie, K., Harwood y, C., Shields, P., *et al.* (2012) Clinically Significant Human Papilloma Virus in Squamous Cell Carcinoma of the Head and Neck in UK Practice *Clinical Oncology* 25: 18-23

Hecht, S.H. (1999) Tobacco smoke carcinogens and lung cancer. *Journal of National Cancer Institute* 91: 1194–1210

Heck, J. E., Sapkota, A., Vendhan, G., Roychowdry, S., Dikshit, R. P., Jetly, D. H. *et al*. (2008) Dietary risk factors for hypopharyngeal cancer in India. *Cancer Causes Control* 19: 1329–37

Heck, J. E., Vaccarella, B. J., Winn, S., Smith, D. M., Shan'gina, E. M., Schwartz, S. M. *et al.* (2010) Sexual behaviours and the risk of head and neck cancers: a pooled analysis in the International Head and Neck Cancer Epidemiology (INHANCE) consortium. *International Journal of Epidemiology* 39: 166–81.

Hemminki, K., Dong, C. and Frisch, M. (2000) Tonsillar and other upper aerodigestive tract cancers among cervical cancer patients and their husbands. *European Journal of Cancer Prevention* 9: 433–437.

Herman, L., Hubert. P., Herfs, M., Kustermans, G., Henrotin, Y. and Bousarghin, L. *et al*. (2010) The L1 major capsid protein of HPV16 differentially modulates APC trafficking according to the vaccination or natural infection context. *European Journal of Immunology* 40: 3075–84

Hernandez, B. Y., Wilkenns, L. R., Zhu, X., Thompson, P., McDuffi, K., Shvetsov, Y. B. *et al*. (2008) Transmission of human papillomavirus in heterosexual couples *Emerging Infectious Diseases* 14: 888-894

Herrero, R., Castellsagué X., Pawlita, M., Lissowska, J., Kee, F., Balaram, P. *et al*. (2003) Human papillomavirus and oral cancer: The International Agency for Research on Cancer multicenter study. *Journal of National Cancer Institute* 95: 1772–83

Hertrampf, K., Eisemann, N., Wiltfang, J., Pritzkuleit, R., Hans-Jürgen, W. and Waldmann, A. (2015) Baseline data of oral and pharyngeal cancer before introducing an oral cancer prevention campaign in Germany *Journal of Cranio-Maxillo-Facial Surgery* 43(3): 360-366

Heusinkveld, M., Goedemans, R., Briet, R. J., Gelderblom, H., Nortier, J. W. and Gorter, A. *et al*. (2012) Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer. *International Journal of Cancer* 131: 74–85.

Hindle, I., Downer, M. C., Moles, D. R. and Speight, P. M. (2000). Is alcohol responsible for more intra-oral cancer? *Oral Oncol*ogy 36: 328–333

Hodges, F. M. (1997) A short history of the institutionalization of involuntary sexual mutilation in the United States. In: Denniston, G., Milos, M. F., editors. Sexual mutilations: A Human Tragedy. *New York, NY: Plenum Press*; pp 17-40

Hong, A. M., Dobbins, T. A., Lee, C. S., Jones, D., Harnett, G. B., Armstrong, B. K. *et al*. (2010) Human papillomavirus predicts outcome in oropharyngeal cancer in patients treated primarily with surgery or radiation therapy. *British Journal of Cancer* 103(10): 1510–7

Hong, A. M., Martin, A., Chatfield, M., Jones, D., Zhang, M., Armstrong, B., *et al*. (2013) Human papillomavirus, smoking status and outcomes in tonsillar squamous cell carcinoma. *International Journal of Cancer* 132: 2748–54.

Honorato, J., Rebelo, M. S., Dias, F. L., Camisasca, D. R., Faria, P. A., Azevedo e Silva, G. *et al*. (2015) Gender differences in prognostic factors for oral cancer *International Journal of Oral and Maxillofacial Surgery* 44(10): 1205-1211

Horton, R. and Das, P. (2011) Male circumcision and HPV transmission to female partners *The Lancet* 377: 183-184

Howe, R. S. V. and Storms, M. R. (2009) Circumcision to prevent HPV infection: Reflection and reaction *The Lancet* 10: 746-747

Howie, N. M., Trigkas, T. K., Cruchley, A. T., Wertz, P. W., Squier, C. A. and Williams, D. M. (2001). Short-term exposure to alcohol increases the permeability of human oral mucosa. *Oral Dis*eases 7: 349–354.

Howlader, N., Noone, A. M. and Krapcho, M. (1975-2008) editor (Online). SEER Cancer Statistics Review, 1975-2008. Bethesda (MD): National Cancer Institute. Available from: http://seer.cancer.gov/csr/1975_2009_pops09/, based on November 2010 SEER data submission, posted to the SEER web site, 2011. [Accessed 02.06.15]

Huang, L. W., Chao, S. L., Chen, P. H. and Chou, H. P. (2004) Multiple HPV genotypes in cervical carcinomas; Improved DNA detection in cervical carcinomas *Journal of Clinical Virology* 29(4): 271-6

Hubert, P., Caberg, J. H., Gilles, C., Bousarghin, L., Franzen-Detrooz, E., Boniver, J., *et al*. (2005) E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in cervical human papillomavirus-associated (pre)neoplastic lesions. *Journal of Pathology* 206: 346–55.

Hughes, G. and Lowndes, C. M. (2014) Epidemiology of sexually transmitted infections: UK review. *Medicine* 42(6): 281-286

Hugo, T. D., Maier, V. T., Jansen, K., Rodrigues, C. E. G., Cruzeiro, A. L. S., Ores, L., *et al.* (2011) Factors associated with age at first intercourse: a population-based study. *Cadernos de Saude Publica* 27: 2207.

Husgavfel-Pursiainen, K. (2004) Genotoxicity of environmental tobacco smoke: A review. *Mutation Research* 567: 427–445.

IACR (2012) International Agency for Research on Cancer [Online] GLOBOCAN UK Available from: http://www-dep.iarc.fr/ [Accessed 01.06.15].

IACR (2012) List of Classifications by cancer sites with sufficient or limited evidence in humans, Volumes 1 to 105*. [Online] International Agency for Research on Cancer. Available from http://monographs.iarc.fr/ENG/Classification/index.php. [Accessed 15.05.15].

IARC (1986) Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, *Tobacco Smoking, Lyon, France*: 38

IARC/WHO: GLOBOCAN (2012) International Agency for Research on Cancer. Available from: http://globocan.iarc.fr,2012; 2014 [Accessed 29.01.14].

Information Services Division Scotland (2016) Cancer Incidence in Scotland [Online] Cancer Information Programme. Available from: www.isdscotland.org/cancer [Accessed 08.08.16].

ISD Scotland (2014) [Online] Available from: http://www.isdscotland.org/Health Topics/Cancer/Publications/index.asp [Accessed 15.01.16].

Jiron, J., Sethi, S., Ali-Fehmi, R., Franceschi, S., Struijk, L., Leen-Jan van Doorn, Q. *et al.* (2014) Racial disparities in Human Papillomavirus (HPV) associated head and neck cancer *American Journal of Otolaryngology – Head and Neck medicine and surgery 35*: 147 – 153

Johnson, A.M., Wadsworth, J., Wellings, K. & Field, J. (1994) *Sexual Attitudes and Lifestyles*. Oxford: Blackwell Scientific Publications

Joseph, A. W. and D'Souza (2012) Epidemiology of Human Papillomavirus-Related Head and Neck Cancer *Otolaryngologic Clinics of North America* 45(4): 739-764

Junquiera, L. C. Joseph, L. J., Goodman, M., Higgins, K., Pilai, R., Ramalingam, S. S. *et al*. (2005), *Basic Histology*, p. 282

Kanodia, S., Fahey, L. M. and Kast, W. M. (2007) Mechanisms used by human papillomaviruses to escape the host immune response. *Current Cancer Drug Targets* 7: 79–89.

Karim-Kos HE, de Vries E, Soerjomataram I, Lemmens, V., Seisling, S. and Coebegh, J. W. (2008) Recent trends of cancer in Europe: a combined approach of

incidence, survival and mortality for 17 cancer sites since the 1990s. *European Journal of Cancer* 44(10): 1345–89.

Kelly, J. R., Hussain, Z. A. and Burtness, B. (2016) Treatment de-intensification strategies for head and neck cancer *European Journal of Cancer* 68: 125-133

Kenyon, C. R., Tsoumanis, A. and Schwartz, I. S. (2016) A population's higher-risk sexual behaviour is associated with its average sexual behaviour: An ecological analysis of subpopulations in Ethiopia, Kenya, South Africa, Uganda and the United States *Epidemics* 15: 56–65

Kero, K., Rautava, J., Syrjanen, K., Grenman, S. and Syrjanen, S (2012) Oral mucosa as a reservoir of human papillomavirus: point prevalence, genotype distribution, and incident infections among males in a 7-year prospective study *European Journal of Urology* 62: 1063–70

Kerr, D. A., Pitman, M. B., Sweeney, B., Arpin, R. N., Wilbur, D. C., Faquin, W. C. (2014) Performance of the Roche Cobas 4800 high-risk human papillomavirus test in cytologic preparations of squamous cell carcinoma of the head and neck. *Cancer Cytopathology* 122: 167-174

Khlifi, R. and Hamza-Chaffai, A. (2010) Head and neck cancer due to heavy metal exposure via tobacco smoking and professional exposure: A review. *Toxicology and Applied Pharmacology* 248: 71–88

Klein, S. L. (2000) The effects of hormones on sex differences in infection: from genes to Behaviour. *Neuroscience and Biobehavioural Reviews* 24: 627–638.

Knepp, J. H., Geahr, M. A., Forman, M. S. and Valsamakis, A. (2003) Comparison of automated and manual nucleic acid extraction methods for detection of enterovirus RNA. *Journal of Clinical Microbiology* 41: 3532–6

Knight, G. L., Needham, L., Ward, D. and Roberts, S. (2016) Pilot study investigating the prevalence of oral Human Papilloma Viral (HPV) infection in young adults *Public Health* 132: 105-107

Koskimaa, H. M., Waterboer ,T., Pawlita. M., Grenman, S., Syrjanen, K. and Syrjanen, S. (2011) Human papillomavirus genotypes present in the oral mucosa of newborns and their concordance with maternal cervical human papillomavirus genotypes. *Journal of Paediatrics* 160: 837–43.

Kreimer, A. R., Alberg, A. J., Daniel, R., Gravitt, P. E., Viscidi, R., Garrett, E. S., *et al.* (2004) Oral human papillomavirus infection in adults is associated with sexual behavior and HIV serostatus. *Journal of Infectious Diseases* 189: 686–698.

Kreimer, A. R., Bhatia, R. K., Messeguer, A. L. *et al*. (2010). Oral human papillomavirus in healthy individuals: a systematic review of the literature. *Sexually Transmitted Diseases* 37: 386–391.

Kreimer, A. R., Clifford, G. M., Boyle, P. and Franceschi, S. (2005). Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiology Biomarkers Preview* 14: 467–475.

Kreimer, A. R., Villa, A., Nyitray, A. G. (2010) International HPV Meeting, Oral Presentation: *Oral HPV Natural History among Healthy Men in the HIM Study*.

Kreimer, A. R., Villa, A., Nyitray, A. G. Abrahamsen, M., Papenfuss, M., Smith D. *et al*. (2011) The epidemiology of oral HPV infection among a multinational sample of healthy men. *Cancer Epidemiology Biomarkers and Prevention* 20(1): 172–82.

Kriek, J. M., Jaumdally, S. Z., Masson, L., Little, F., Mbulawa, Z., Gumbi, P. P. *et al*. (2016) Female genital tract inflammation, HIV co-infection and persistent mucosal Human Papillomavirus (HPV) infections *Virology* 493: 247-254

Krüger, M., Pabst, A. M., Walter, C., Sagheb, K., Gunther, C., Blatt, S. *et al*. (2014) The prevalence of human papilloma virus (HPV) infections in oral squamous cell carcinomas; a retrospective analysis of 88 patients and literature overview. *Journal of Craniomaxillofacial Surgery* 42: 1506-1514

Kumar, B., Cordell, K. G., Lee, J. S., Prince, M. E., Tran, H. H., Wolf, G. T. *et al.* (2007) Response to Therapy and Outcome in Oropharyngeal Cancer are Associated with Biomarkers Including HPV, EGFR, Gender and Smoking *International Journal of Radiation Oncology Biology Physics* 69 (2): s109-s111

Lajer, C. B., Garnæs, E., Friis-Hansen, L., Norrild. B., Therkildsen, M. H., Glud, M. *et al*. (2012) The role of miRNAs in human papilloma virus (HPV)-associated cancers: bridging between HPV-related head and neck cancer and cervical cancer *British Journal of Cancer* 106: 1526–1534

Langendijk, J. A., Doornaert, P., Verdonck-de Leeuw, I. M., Leemans, C. R., Aaronson, N. K. and Slotman, B. J. (2008) Impact of late treatment-related toxicity on quality of life among patients with head and neck cancer treated with radiotherapy. *Journal of Clinical Oncol*ogy 26(22): 3770–6.

Lassen P, Eriksen, J. G., Hamilton-Dutoit, S., Tramm, T., Alsner, J. and Overgaard, J. (2009) Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *Journal of Clinical Oncol*ogy 27(12): 1992–8.

Lassen, P., Eriksen, J. G., Krogdahl, A., Therkildsen, M. H., Ulhoi, B. P., Overgaard, M. *et al*. (2011) The influence of HPV-associated p16-expression on accelerated fractionated radiotherapy in head and neck cancer: evaluation of the randomised DAHANCA 6&7 trial. *Radiotherapy and Oncology* 100(1): 49–55

Laus, S., Kingsley, L. A., Green, M. and Wadowsky, R. M. (2011) Comparison of QIAsymphony Automated and QIAamp Manual DNA Extraction Systems for Measuring Epstein-Barr Virus DNA Load in Whole Blood Using Real-Time PCR *The Journal of Molecular Diagnostics* 13(6): 695-700

Lee, J., Taneja, V. and Vassalo, R. (2012) Cigarette smoking and inflammation: *Cellular and Molecular Mechanisms*. 91:142–9

Leichliter, J. S., Chandra, A., Liddon, N. and Fenton, K. A., Aral, S. O. (2007). Prevalence and correlates of heterosexual anal and oral sex in adolescents and adults in the United States. *Journal Infectious Diseases* 196: 1852–9.

Leigh, B. C., Temple, M. T. and Trocki, K. F (1993) The sexual behaviour of US adults: results from a national survey. *American Journal of Public Health* 83: 1400–8

Li, G. and Sturgis, E. M. (2006) The role of human papillomavirus in squamous carcinoma of the head and neck. *Current Oncology Rep*orts 8: 130–139.

Licitra, L., Perrone, F., Bossi, P., Suardi, S., Mariani, L., Artusi, R. *et al*. (2006) High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *Journal of Clinical Oncol*ogy 24(36): 5630–6

Lima, M. D. M., Braz-Silva, P. H., Pereira, S. M., Riera, C., Coelho, A. C. and Gallottini, M. (2014) Oral and cervical HPV infection in HIV-positive and HIV-

negative women attending a sexual health clinic in São Paulo, Brazil. *International Journal of Gynecology and Obstetrics* 126: 33–36.

Lindel, K., Beer, K. T., Laissue, J., Greiner, R. H., Aebersold, D. M. (2001) Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. *Cancer* 92(4): 805–13

Lissowska, J. (2003) Smoking, alcohol, diet, dentition and sexual practices in the epidemiology of oral cancer in Poland, *European Journal of Cancer Prevention* 12 (1): 25–33.

Little, J., Cardy, A. and Munger, R.G. (2004) Tobacco smoking and oral clefts: a meta-analysis. Bull. *World Health Organ*. 82: 213–218.

Lloyd, J., Crouch, N. S., Minto, C. L. Liao, L. M and Creighton, S. M. (2005) "Female genital appearance: 'normality' unfolds" *British Journal of Obstetrics and Gynaecology* 112(5): 643–646

Lopes, V., Murray, P., Williams, H., Woodman, C., Watkinson, J. and Robinson, M. (2011) Squamous cell carcinoma of the oral cavity rarely harbours oncogenic human papillomavirus *Oral Oncology* 47(8): 698-701

Louie, K. S., Mehanna, H. and Sasieni, P (2015) Trends in head and neck cancers in England from 1995 to 2011 and projections up to 2025 *Oral Oncology* 51: 341–348

Maden, C. (1992) Human papillomaviruses: herpes simplex viruses, and the risk of oral cancer in men *American Journal of Epidemiology* 135 (10): 1093–1102.

Mahal, B. A., Inverso, I., Aizer, A. A., Donoff, R. B. and Sung-Kiang Chuang (2014) Impact of African–American race on presentation, treatment, and survival of head and neck cancer *Oral Oncology* 50: 1177-1181

Mallen-St Clair, J., Alani, A., Wang, M. B. and Srivastan, E. S. (2016) Human papillomavirus in oropharyngeal cancer: The changing face of a disease *Biochimica et Biophysica Acta* 1866: 141–150

Marcu, L. G. (2016) Future treatment directions for HPV-associated head and neck cancer based on radiobiological rationale and current clinical evidence *Critical Reviews in Oncology/ Hematology* 103: 27–36

Markle, W., Conti, T. and Kad, M. (2013) Sexually Transmitted Diseases Review Primary Care: *Clinics in Office Practice* 40: (3) 557-587

Marur, S., D'Souza, G., Westra, W. H, Forastiere, A. A. (2010) HPV-associated head and neck cancer: a virus-related cancer epidemic. *Lancet Oncology* 11(8):781–9.

Mathers, C. D., Fat, D. M., Inoue, M., Rao, C. and Lopez, A. D. (2005) Counting the dead and what they died from: an assessment of the global status of cause of death data *Bulletin of the World Health Organization* 83:171-177.

Matsushita, K., Sasagawa T, Miyashita, M., *et al*. (2011) Oral and cervical human papillomavirus infection among female sex workers in Japan. *Japanese Journal of Infectious Diseases* 64(1): 34–9.

Maxwell, J. H., Kumar, B., Feng, F. Y., Worden, F. P., Lee, J. S, Eisbruch A. *et al*. (2010) Tobacco use in human papillomavirus-positive advanced oropharynx cancer patients related to increased risk of distant metastases and tumour recurrence. *Clinical Cancer Research* 16: 1226–35.

McKaig, R. G., Baric, R. S. and Olshan, A. F. (1998) Human Papillomavirus and head and neck cancer: Epidemiology and molecular biology *Head and Neck* 20(3): 250-265

Mehanna, H., Beech, T., Nicholson, T., El-Hariry, I., McConkey, C., Paleri, V. *et al*. (2013) Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal head and neck cancer—systematic review and metaanalysis of trends by time and region, *Head and Neck* 35(5): 747–755

Mellin, H., Friesland, S., Lewensohn, R., Dalianis, T. and Munck-Wikland, E. (2000) Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse, and survival. *International Journal of Cancer.* 89(3): 300–4

Mercer, C. H. (2014) Sexual behaviour *Epidemiology and Sexual Behaviour* 42: 6

Merletti F, Boffetta P, Ciccone G, Mashberg A, Terracini B. (1989) Role of tobacco and alcoholic beverages in the etiology of cancer of the oral cavity/oropharynx in Torino, Italy. *Cancer Research* 49: 4919-4924.

Milgrom, P., Tut, O. K., Gallen, M., Mancl, L., Spillane, N., Chi, D. L. *et al.* (2016) Symptoms with betel nut and betel nut with tobacco among Micronesian youth *Addictive Behaviours* 53: 120–124

Montero, P. H., and Patel, S. G. (2015) Cancer of the Oral Cavity *Surgical Oncology Clinics of North America* 24(3): 491-508

Mooij, S. H ., Boot, H. J., Speksnijder, A. G., Stolte, I. G., Meijer, C. J., Snijders P. J. *et al*. (2013) Oral human papillomavirus infection in HIV-negative and HIV-infected men who have sex with men: the HIV & HPV in MSM (H2M) study. *AIDS Lippincott Williams and Wilkins* 1473-5571

Moreno-Ribera, N., Fuertes-de Vega, I., Blanco-Arévalo, J. L., Bosch-Mestres, J., González-Cordón, A., Estrach-Panella, T. *et al*. (2016) Sexually Transmitted Infections: Experience in a Multidisciplinary Clinic in a Tertiary Hospital (2010-2013) *Actas Dermo-Sifiliográficas* 107(3): 235-241

Mork, J. and Glattre, E. (1998) Squamous cell carcinomas of the head and neck in Norway, 1953-92: an epidemiologic study of a low-risk population. *Cancer Causes Control* 9(1): 37–48.

Mosher, W. D., Chandra, A. and Jones, J. (2002) Sexual behavior and selected health measures: men and women 15–44 years of age, United States, 2002. Advance data from vital and health statistics; number 362. Hyattsville, MD: *National Center for Health Statistics*; 2005.

Mourad, W. F., Hu, K. S., Shasha, D., Concert, C. M., Ryniak, M., Shourbaji, R. A. *et al*. (2011) The Impact of HPV, HIV, and Smoking on Patients With Oropharynx Cancer and Metastases of Unknown Primary of the Head and Neck *International Journal of Radiation Oncology Biology Physics* 93(3): E328

Myers, J. N., Elkins, T., Roberts, D. and Byers, R. M. (2000) Squamous cell carcinoma of the tongue in young adults: increasing incidence and factors that predict treatment outcomes. *Otolaryngology Head Neck Surgery* 122: 44–51.

Nanci (2013) *Ten Cate's Oral Histology* Elsevier pp 280 NCIN Potentially HPV-related head and neck cancers (2012) *NCIN Data Briefing*; May 2012

Nasman, A., Attner, P., Hammarstedt, L., Du, J., Eriksson, M and Giraud, G, *et al*., (2009) Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in

Stockholm, Sweden: an epidemic of viral-induced carcinoma *International Journal of Cancer* 125: 362–366

NATSAL UK (2010-2012) The National Survey of Sexual Attitudes and Lifestyles [Online] NATSAL UK. Available from: http://www.natsal.ac.uk/media/2102/natsal-infographic.pdf http://www.natsal.ac.uk/home.aspx [Accessed 08.06.16]

Nik Farid, N. D., Che'Rus, S., Dahlui, M. and Al-Sadat, N. (2013) Determinants of sexual intercourse initiation among incarcerated adolescents: a mixed-method study. *Singapore Medical Journal* 54: 695

Nofech-Mozes, S., Khalifa, M. M., Ismiil, N., Dubé, V., Saad, R. S., Sun, P. *et al*. (2010). Detection of HPV-DNA by a PCR-based method in formalin-fixed, paraffin-embedded tissue from rare endocervical carcinoma types. *Applied Immunohistochemistry and Molecular Morphology* 18(1): 80-85

Norat, T., Scoccianti, C., Boutron-Ruault, M., Anderson, A., Berrino, F., Cecchini *et al.* (2015) European Code against Cancer 4th Edition: Diet and cancer *Cancer Epidemiology* 39: S56–S66

O'Rorke, M. A., Ellison, M.V., Murray, L. J., Moran, M., James, J., Anderson, L. A. (2012) Human papillomavirus related head and neck cancer survival: a systematic review and meta-analysis. *Oral Oncology* 48(12): 1191–201

Office for National Statistics. Cancer Statistics: Registrations Series MB1. http://www.statistics.gov.uk/statbase/Product.asp?vlnk=8843.

Ogden, G. R. (2005) Alcohol and oral cancer *Alcohol* 35: 169–173

Ogden, G. R. and Graham, C. (2003). Mouth cancer awareness in Dundee, Scotland: public perceptions and attitudes. In A. K. Varma, & P. Reade (Eds.), Oral Oncology (Volume 9, Proceedings of the 9th *International Congress on Oral Cancer*, November 2003, Melbourne, Australia; pp. 9–17). New Delhi: Rajiv Beri for Macmillan India Ltd.

Olesen, T. B., Jensen, K.E., Nygard, M., Tryggvadottir, L., Sparén, P., Hansen, B. T. *et al*. (2012) Young age at first intercourse and risk-taking behaviours. A study of nearly 65 000 women in four Nordic countries *European Journal of Public Health* 22: 220

Osazuwa-Peters, N., Wang, D. D., Namin, A., John,V. M. M., O'Neill , M., Patel, P. V. *et al*. (2015) Sexual behaviour, HPV knowledge, and association with head and neck cancer among a high-risk group *Oral Oncology* 51: 452–456

Osazuwa-Peters, N., Wang, D. D., Namin, A., John, V. M., O'Neill, M., Patel, P. V. and Varvares, M. A. (2015) Sexual behaviour, HPV knowledge, and association with head and neck cancer among a high-risk group *Oral Oncology* 51: 452–456

Palefsky, J. M., Holly, E. A., Ralston, M. L., Da Costa, M. and Greenblatt, R. M. (2001) Prevalence and risk factors for anal human papillomavirus infection in human immunodeficiency virus (HIV)-positive and high-risk HIV-negative women. *Journal of Infectious Diseases* 183: 383–91

Pallant, J. (2013) A step by step guide to data analysis using IBM SPSS. *SPSS Survival Manual* 5th Edition pp 154-186

Palmer, E., Newcombe, R. G., Green, A. C., Kelly, C., Gill, O. N., Hall, G. *et al*., (2014) Human papillomavirus infection is rare in nonmalignant tonsil tissue in

the UK: implications for tonsil cancer precursor lesions, *International Journal of Cancer* 135 (10): 2437–2443

Palmer, R. M., Wilson, R. F., Hasan, A.S. and Scott, D. A. (2005) Mechanisms of action of environmental factors – tobacco smoking. *Journal of Clinical Periodontology* 32: 180–95

Pandeya, N., Olsen, C. M. and Whiteman, D. C. (2013) Sex differences in the proportion of esophageal squamous cell carcinoma cases attributable to tobacco smoking and alcohol consumption. *Cancer Epidemiology* 592: 6

Pannone, G., Santoro, A., Papagerakis, P., Muzio, L. L., De Ros, G. and Pantaleo Bufo *et al*. (2011) *Infectious Agents and Cancer* 6: 4

Pappas, R. S., Polzin, G. M., Zhang, L., Watson, C. H., Paschal, D. C., Ashley, D. L. (2006) Cadmium, Lead and thallium in Mainstream tobacco smoke particulate. *Food and Chemical Toxicology* 44: 714–723.

Parisi, S. G., Cruciani, M., Scaggiante, R., Boldrin, C., Andreis, S., Bello, F. D. *et al*. (2011) Anal and oral human papillomavirus (HPV) infection in HIV-infected subjects in northern Italy: a longitudinal cohort study among men who have sex with men. *BioMed Central Infectious Disease* 11: 150.

Park, J. M., Jung, C. K., Choi, Y. J., Lee, K. Y., Kang, J. H., Kim, M. S. *et al*. (2010) The use of an immunohistochemical diagnostic panel to determine the primary site of cervical lymph node metastases of occult squamous cell carcinoma. *Human Pathology* 41(3): 431–7.

Parkin, D. M. (2011) *British Journal of Cancer* 105 (S2): S49-S56

Patel, S. C., Carpenter, W. R., Tyree, S., Couch, M. E., Weissler, M., Hackman, T. *et al*. (2011) Increasing incidence of oral tongue squamous cell carcinoma in young white women, age 18 to 44 years *Journal of Clinical Oncology* 29(11): 1488-94.

Peralta, R., Baudis, M., Vazquez, G., Juarez, S., Ortiz, R., Decanini, H. *et al*. (2010) Increased expression of cellular retinolbinding protein 1 in laryngeal squamous cell carcinoma. *Journal of Cancer Research Clinical Oncology* 136(6): 931–8

Petersen, P. E. (2009) Oral cancer prevention and control – The approach of the World Health Organization *Oral Oncology* 45(4-5): 454-460

Petti a, S., Mohd, M and Scully, C. (2012) Revisiting the association between alcohol drinking and oral cancer in non-smoking and betel quid non-chewing individuals *Cancer Epidemiology* 36: e1–e6

Petti, S. (2009) Lifestyle risk factors for oral cancer *Oral Oncology* 45(4–5): 340-350

Pickard, R. K., Xiao, W., Broutian, T. R., He, X. and Gillison, M. L. (2012) The prevalence and incidence of oral human papillomavirus infection among young men and women, aged 18–30 years. *Sexually Transmitted Diseases* 39: 559–6

Pintos, J., Black, M. J., Sadeghi, N., Ghadirian, P., Zeitouni, A. G., Viscidi, R. P., Herrero, R., Coutlée, F. and Franco, E. L. (2008) Human papillomavirus infection and oral cancer: A case-control study in Montreal, Canada *Oral Oncology*, 44(3): 242-250

Pista, A., de Oliveira, C. F., Cunha, M. J., Paixao, M. T. and Real, O. (2012) CLEOPATRE Portugal Study Group 1 Risk factors for human papillomavirus infection among women in Portugal: The CLEOPATRE Portugal Study. *International Journal of Gynecology and Obstetrics* 118: 112–116

Polesel, J., Franceschi, S., Talamini, R., Negri, E., Barzan, L., Montella, M. *et al*. (2011) Tobacco smoking, alcohol drinking, and the risk of different histological types of nasopharyngeal cancer in a low-risk population *Oral Oncology* 47: 541–545

Pons-Salort, M., Letort, V., Favre, M., Heard, I., Dervaux, B., Opatowski, L. *et al*. (2013) Exploring individual HPV co-infections is essential to predict HPV-vaccination impact on genotype distribution: a model-based approach. *Vaccine* 31(8): 1238-45.

Posner, M. R., Lorch, J. H., Goloubeva, O., Tan, M., Schumaker, L. M., Sarlis, N. J. *et al*. (2011) Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. *Annals of Oncology* 22(5): 1071–7

Prime Minister's Strategy Unit. (2004) Alcohol Harm Reduction Strategy for England. London: Author. Available at http://www.strategy.gov.uk/downloads/su/alcohol/alcohol_harm_pdf.htm.

Quiros, B., Alemany, L., Lloveras, B., Quint, W., Kleter, B., Alejo, M. *et al*. (2010) Comparison of human papillomavirus detection between freshly frozen tissue and paraffin embedded tissue of invasive cervical cancer *Infectious Agents and Cancer* 5: 15

Quon, H. and Richmon, J. D. (2012) Treatment deintensification strategies for HPV associated head and neck carcinomas *Otolaryngologic Clinics of North America* 45(4): 845–61

Ragin, C. C., Langevin, S. M., Marzouk, M., Grandis, J. and Taioli, E. (2011) Determinants of head and neck cancer survival by race. *Head Neck* 33: 1092–8

Ragin, C. C., Modugno, F. and Gollin, S. M. (2007) The epidemiology and risk factors of head and neck cancer: A focus on human papillomavirus. *Journal of Dental Research* 86: 104–14.

Rintala, M. A., Grenman, S. E., Jarvenkyla, M. E., Syrjänen, K. J. and Syrjänen, S. M. (2005) High-risk types of human papillomavirus (HPV) DNA in oral and genital mucosa of infants during their first 3 years of life: experience from the Finnish HPV Family Study *Clinical Infectious Diseases* 41(12): 1728–33.

Rintala, M., Grenman, S., Puranen, M. and Syrjänen, S. (2006) Natural history of oral papillomavirus infections in spouses: a prospective Finnish HPV Family Study. *Journal of Clinical Virology* 35: 89–94.

Rintala, M.A., Grenman, S.E., Jarvenkyla, M.E. (2005) High-risk types of human papillomavirus (HPV) DNA in oral and genital mucosa of infants during their first 3 years of life: experience from the Finnish HPV Family Study. *Clinical Infectious Diseases* 41: 1728–1733.

Rischin, D., Young, R. J., Fisher, R., Fox, S. B., Le, Q. T., Peters, L. J. *et al*. (2010) Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. *Journal of Clinical Oncology* 28(27): 4142–8

Romanowski, B. (2011) Long term protection against cervical infection with the human papillomavirus: review of currently available vaccines. *Human Vaccin*e *and Immunotherapy* 7.

Rosenquist, S. E. (2016) Is Oral Sex Really a Dangerous Carcinogen? Let's Take a Closer Look *The Journal of Sexual Medicine* 13 (7): 1013-1146

Roteli-Martins, C. M., Naud, P., De Borba, P., Teixeira, J. C., De Carvalho, N. S., Zahaf, T. *et al*. (2012) Sustained immunogenicity and efficacy of the HPV-16/18 AS04-adjuvanted vaccine: up to 8.4 years of follow-up. *Human Vaccine and Immunotherapy* 8(3): 390-7.

Ruatava, J., Kuuskoskib, J., Syrjänenc, K., Grenmanb, R. and Syrjänena, S. (2012) HPV genotypes and their prognostic significance in head and neck squamous cell carcinomas *Journal of Clinical Virology* 53: 116– 120

Rubenstein, L. M., Smith, E. M., Pawlita, M., Haugen, T. H., Hamsikova, E., Turek, L. P. (2011) Human papillomavirus serologic follow-up response and relationship to survival in head and neck cancer: a case-comparison study. *Infectious Agents and Cancer* 6: 9

Ryerson, A. B., Peters, E. S., Coughlin, S. S., Chen, V. W., Gillison, M. L., Reichman, M. E. *et al*. (2008). Burden of potentially human papillomavirus-associated cancers of the oropharynx and oral cavity in the US, 1998–2003 *Cancer* 15: 113

Rysavy, M. B., Kresowik, J. D. K., Liu, D., Mains, L., Lessard, M. and Ryan, G. L., (2014) Human Papillomavirus Vaccination and Sexual Behavior in Young Women *Journal of Paediatric Adolescence Gynaecology* 27: 67e7

Şahiner, F., Kubar, A., Yapar, M., Şener, K., Dede, M. and Gümral, R. (2014) Detection of major HPVs by a new multiplex real-time PCR assay using type-specific primers *Journal of Microbiological Methods* 97: 44–50

Salhan, S. (2011) *Textbook of Gynecology* JP Medical Ltd. pp. 270

Samek, D. R., Iacono, W. G., Keyes, M. A., Epstein, M., Bornovalova, M. A.and McGue, M. (2014) The developmental progression of age 14 behavioural disinhibition, early age of sexual initiation, and subsequent sexual risk-taking behaviour. *Journal of Child Psychology and Psychiatry* 55:784

Sand, L. and Jalouli, J. (2014) Viruses and oral cancer. Is there a link? *Microbes and Infection* 16: 371-378

Saraiya, M., Rosser, J. I., Cooper, C. P. (2012) Cancers that U.S. physicians believe the HPV vaccine prevents: findings from a physician survey, 2009. *Journal of Women's Health* 21: 111–117

Sasco, A. J., Secretana, M. B., Straif, K. (2004). Tobacco smoking and cancer: a brief review of recent epidemiological evidence. *Lung Cancer* 45(2): S3–S9

Scherly, D., Nouspikel, T., Corlet, J., Ucla, C., Bairoch, A. and Clarkson, S. G. (1993) Complementation of the DNA repair defect in xeroderma pigmentosum group G cells by a human cDNA related to yeast RAD2. *Nature* 363: 182–5.

Schlecht, N. F., Franco, E. L., Pintos, J., Negassa, A., Kowalski, L. P., Oliveira, B. V. *et al*. (1999) Interaction between tobacco and alcohol consumption and the risk of cancers of the upper aerodigestive tract in Brazil. *American Journal of Epidemiology* 150: 1129-1137

Schoen, E. J. (2007) Circumcision as a lifetime vaccination with many benefits. *The Journal of Men's Health & Gender* 4(3): 306-311

Schoen, E. J., Wiswell, T. L. and Moses, S. (2000) New Policy on Circumcision-Cause for Concern *Paediatrics* 105: 3

Schrank, T. P., Han, Y., Weiss, H. and Resto, V. A. (2011) Case-matching analysis of head and neck squamous cell carcinoma in racial and ethnic minorities in the United States–possible role for human papillomavirus in survival disparities. *Head and Neck* 33(1): 45–53

Schwartz, S. R., Yueh, B., McDougall, J. K., Daling, J. R. and Schwartz, S. M. (2001) Human papillomavirus infection and survival in oral squamous cell cancer: a population-based study. *Otolaryngology Head and Neck Surgery* 125(1): 1–9

Schwartz, S., Daling, J., Doody, D., Wipf, G. C., Carter, J. J., Madeleine, M. M. *et al*. (1998) Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. *Journal of National Cancer Institute* 90: 1625–36.

Scully, C. and Bedi, R. (2000) Ethnicity and oral cancer *The Lancet Oncology* 1

Senkul, T., Iseri, C., Sen, B., Karademir, K., Saacoglu, F. and Erden, D. (2004) Circumcision in adults: effects on sexual function *Urology* 63: 155-158

Settle, K., Posner, M. R., Schumaker, L. M., Tan, M., Suntharalingam, M., Goloubeva, O. *et al.* (2009) Racial survival disparity in head and neck cancer results from low prevalence of human papillomavirus infection in black oropharyngeal cancer patients. *Cancer Prevention Research (Philadelphia)* 2(9): 776–81.

Shaikh, M. H., McMillsn, N. A., Johnson, N. W. (2015) HPV-associated head and neck cancers in the Asia Pacific: A critical literature review & meta-analysis Cancer Epidemiology 39(6): 923-38

Shaikha, M. H., McMillan, N. A. J. and Johnsonc, N. W. (2015) HPV-associated head and neck cancers in the Asia Pacific: A critical literature review & meta-analysis *Cancer Epidemiology* 39: 923–938

Shiboski, C. H., Schmidt, B. L. and Jordan, R. C. (2005) Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20–44 years. *Cancer* 103: 1843–1849.

Shin, D. M., Saba, N. F. (2015) Racial disparities in squamous cell carcinoma of the oral tongue among women: A SEER data analysis *Oral Oncology* 51: 586–592

Simbayi, L. C. (2015) Sexual Risk Behaviours *International Encyclopedia of the Social and Behavioural Sciences (Second Edition)* pp 797–801

Smith, E. M., Ritchie, J. M., Summersgill, K. F, Klussmann, J. P., Lee, J. H, Wang, D. *et al*. (2004) Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. *International Journal of Cancer* 108: 766–772.

Smith, E. M., Rubenstein, L. M., Haugen, T. H., Hamsikova, E., Turek, L. P. (2010) Tobacco and alcohol use increases the risk of both HPV-associated and HPV-independent head and neck cancers. *Cancer Causes Control*. 21: 1369–78.

Smith, E. M., Swarnavel, S., Ritchie, J. M., Wang, D., Haugen, T. H. and Turek, L. P. (2007) Prevalence of human papillomavirus in the oral cavity/oropharynx in a large population of children and adolescents. *Paediatrics Infectious Diseases Journal* 26: 836–840.

Snow, A. N. and Laudadio, J. (2010) Human papillomavirus detection in head and neck squamous cell carcinomas. *Advances in Anatomic Pathology Journal* 17(6): 394–403.

Stanley, M. A. (2006) Immune responses to human papillomavirus. *Vaccine* 24: 16–22.

Stanley, M. A. (2009) Immune responses to human papillomaviruses. *Indian Journal of Medical Research* 130: 266–76.

Stell and Maran's *Textbook of Head and Neck Surgery and Oncology* Fifth Edition (2012) pp 9-11

Stock, M. L. and Paterson, M. L. (2013) Influence on Oral Sex and Oral Cancer Information on young adults Oral sexual risk cognitions and likelihood of HPV vaccination *Journal of sex research* 50(1): 95-102

Sturgis, E. M and Cinciripini, P. M. (2007) Trends in Head and Neck Cancer Incidence in Relation to Smoking Prevalence An Emerging Epidemic of Human Papillomavirus-Associated Cancers? *Cancer* 110(7): 1429-35

Sturgis, E. M., Wei, Q. and Spitz, M. R. (2004) Descriptive epidemiology and risk factors for head and neck cancer. *Seminars in Oncol*ogy 31(6): 726–733

Suarez, C. D., Barnes, L., Silver, C. E., Rodrigo, J. P., Shah, J. P., Triantafyllou, A. et al. (2016) Cervical lymph node metastasis in adenoid cystic carcinoma of oral cavity and oropharynx: A collective international review. *Auris Nasus Larynx* 43(6): 595-718

Sun, Y., Zhang, Y., Limei, L., Song, X. and Li, G. (2015) Genetic polymorphisms and HPV infection in oral squamous cell carcinomas *Current Opinion in Virology* 14: 1–6

Sung, H. L, Chang-Ryul, L., Nicole, K. R., Reuben, H. K., Mo, K. K., No, H. P. *et al*. (2015) Human papillomavirus 16 (HPV16) enhances tumour growth and cancer stemness of HPV-negative oral/oropharyngeal squamous cell carcinoma cells via miR-181 regulation *Papillomavirus Research* 1(12): 116-125

Syrjanen, S. (2004) HPV infections and tonsillar carcinoma. *Journal of Clinical Pathology* 57: 449–455

Syrjanen, S., Lodi, G., von Bültzingslöwen, I., Aliko, A., Arduino, P., Campisi G. *et al* (2011) Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review *Oral Diseases* 17(1): 58-72

Tabachnick, B. G. and Fidell, L. S. (2013). *Using multivariate statistics* (6th edition). Boston: Pearson Education.

Tachezy, R., Klozar, J., Rubenstein, L., Smith, E., Salakova, M., Smahelova, J. *et al*. (2009) Demographic and risk factors in patients with head and neck tumours. *Journal of Medical Virology* 81(5): 878–887

Tarnaud, C., Pascale, L., Ewalde, C., Adrian, P., Dirk, T., and Bertran, A. (2011) Association of Low-Risk Human Papillomavirus Infection with Male Circumcision in Young Men: Results from a Longitudinal Study Conducted in Orange Farm (South Africa) *Infectious Diseases in Obstetrics and Gynecology*

Tarvainen, L., Suuronen, R., Lindqvist, C. and Malila, N. (2004) Is the incidence of oral and pharyngeal cancer increasing in Finland? An epidemiological study of 17 383 cases in 1953–1999. *Oral Diseases* 10: 167–172

Tataru, D., Mark, V., Simo, R., Davies, E. A. and Gallagher, J. E. (2016) Trends in Epidemiology of Head and Neck Cancer in London. *Clinical Otolaryngology* 10: 1749

Tay, S. K. and Oon, L. L. (2014) Prevalence of cervical human papillomavirus infection in healthy women is related to sexual behaviours and educational level: a cross-

sectional study. *International Journal of Sexually Transmitted Disease AIDS* 25(14): 1013–21

Termine, N., Giovannelli, L. and Matranga, D. (2011) Oral human papillomavirus infection in women with cervical HPV infection: new data from an Italian cohort and a metanalysis of the literature. *Oral Oncology* 47: 244–250.

Tilahun, M. and Ayele, G (2013) Factors associated with age at first sexual initiation among youths in Gamo Gofa, south west Ethiopia: a cross sectional study. *BMC Public Health* 13: 622

UNDESA (2004) Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat/World population prospects: the 2004 revision and urbanization prospects: Population Division of Department of Economic and Social Affairs of the United Nation Secritariat.

United States Census 2010. http://www.census.gov/

Vajdic, C. M., McDonald, S. P., McCredie, M. R., van Leeuwen, M. T., Stewart, J. H., Law, M. *et al*. (2006) Cancer incidence before and after kidney transplantation. *Journal of American Medical Association* 296(23): 2823–31

Van Dijk, B. A. C., Gatta, G., Capocaccia, R., Pierannunzio, D., Strojan, P., Licitra, L. (2012) The RARECARE Working Group Rare cancers of the head and neck area in Europe *European Journal of Cancer* 48: 783 – 796

van Monsjou, H. S., Wreesmann, V. B., van den Brekel, M. W. M and Balm, A. J. M. (2013) Head and neck squamous cell carcinoma in young patients *Oral Oncology* 49: 1097–1102

van Zanten, E., Wisselink, G. J., de Boer, W., Stoll, S., Alvarez, R. and Kooistra-Smid, A.M.D. (2011) Comparison of the QIAsymphony automated nucleic acid extraction and PCR setup platforms with NucliSens easyMAG and Corbett CAS-1200 liquid handling station for the detection of enteric pathogens in faecal samples *Journal of Microbiological Methods* 84: 335–340

Venuti, A., Paolini, F., Nasir, L., Corteggio, A., Roperto, S., Campo, M. S. *et al*. (2011) Papillomavirus E5: The smallest oncoprotein with many functions. *Molecular Cancer* 10: 1–18.

Verheyena, J., Kaiser,R., Bozic, M., Timmen-Wegoa, M., Maier, B. K., and Kessler, H. H. (2012) Extraction of viral nucleic acids: Comparison of five automated nucleic acid extraction platforms *Journal of Clinical Virology* 54: 255–259

Wang, X. I., Thomas, J. and Zhang, S. (2012) Changing trends in human papillomavirus-associated head and neck squamous cell carcinoma. *Annals of Diagnostic Pathology* 16:7–12.

Wansom, D., Light, E., Thomas, D., Worden, F., Prince, M., Urba, S. *et al*. (2012) Infiltrating lymphocytes and human papillomavirus-16–associated oropharyngeal cancer. *Laryngoscope* 122: 121–7.

Wansom, D., Light, E., Worden, F., Prince, M., Urba, S., Chepeha, D. B. *et al*. (2010) Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. *Archives of Otolaryngology Head Neck Surgery* 136: 1267–73

WCRF, (1997) World Cancer Research Fund Ancan Institute for Cancer Research, Food, Nutrition and Prevention of Cancer: a Global Perspective. *American Institute of Cancer Research*, Washington.

Weinberger, P. M., Yu, Z., Haffty, B. G., Kowalski, D., Harigopal, M., Sasaki, C. *et al*. (2004) Prognostic significance of p16 protein levels in oropharyngeal squamous cell cancer. *Clinical Cancer Research* 10(17): 5684–91.

Weinberger, P. M., Yu, Z., Haffty, B. G., Kowalski, D., Harigopal, M., Brandsma, J. *et al*. (2004) Molecular classification identifies a subset of human papillomavirus–associated oropharyngeal cancers with favourable prognosis. *Journal of Clinical Oncology* 24(5): 736–47.

Weinberger, P. M., Yu, Z., Kountourakis, P., Sasaki, C., Haffty, B. G., Kowalski, D. *et al*. (2009) Defining molecular phenotypes of human papillomavirus associated oropharyngeal squamous cell carcinoma: validation of three-class hypothesis. *Otolaryngology Head Neck Surgery* 141: 382–9.

Wellings, K., Field, J., Johnson, A.M., Wadsworth, J. and Bradshaw, S. (1994) *Sexual Behaviour in Britain: The National Survey of Sexual Attitudes and Lifestyles.* London: Penguin Books Ltd

Welsh Cancer Intelligence and Surveillance Unit (2014) Cancer in Wales. Available from: http://www.wales.nhs.uk/sites3/page.cfm?orgid=242&pid=59080 [Accessed 10.08.16]. Welsh Cancer Intelligence and Surveillance Unit. http://www.wcisu.wales.nhs.uk

WHO (2002) The World Health Reported 2002, Reducing Risks, Promoting Health Life. W.H.O., Geneva, Switzerland. WHO (www.who.int/healthinfo/en/)

Worden, F. P., Kumar, B., Lee, J. S.,Wolf, G. T., Cordell, K. G., Taylor, J. M. *et al*. (2008) Chemoselection as a strategy for organ preservation in advanced oropharynx cancer: response and survival positively associated with HPV16 copy number. *Journal of Clinical Oncology* 26(19): 3138–46.

World Report (2013) Studying sexual health in the UK. *The Lancet* 382: 30

Wynder, E. L., Bross, I. J., Feldman, R. M. (1957) A study of the etiologic factors in cancer of the mouth *Cancer.* 10: 1300–1323.

Wynder, E. L., Graham, E. A. (1950) Tobacco smoking as a possible etiologic factor in bronchiogenic carcinoma, A study of 684 proved cases. *Journal of American Medical Association.* 143: 329–336.

Yang, G., Erdman, D. E., Kodani, M., Kools, J., Bowen, M. D. and Fields, B. S (2011) Comparison of commercial systems for extraction of nucleic acids from DNA/RNA respiratory pathogens. *Journal Virology Methods* 171: 195–9

Young, D., Xiao, C. C., Murphy, B., Moore, M., Fakhry, C. and Day, T. A. (2015) Increase in head and neck cancer in younger patients due to human papillomavirus (HPV) *Oral Oncology* 51: 727–730

Yu, B., Nagarajan, V. K. and Ferris D. G. (2015) Mobile fiber optic sensor for Detection of oral and cervical cancer in the developing world. Mobile health technologies: Methods and Protocols *Methods in Molecular Biology* 1256

Zakeri, K., MacEwan, I., Vazirnia, A., Cohen, E. E. W., Spiotto, M. S., Haraf, D. J. *et al.* (2014) Race and competing mortality in advanced head and neck cancer *Oral Oncology* 50: 40–44

Zhang, Y., Waterboer, T., Pawlita, M., Sugar, E., Minkoff, H., Cranston, R. D. *et al*. (2016) Human Papillomavirus (HPV) 16 E6 seropositivity is elevated in subjects with oral HPV16 infection. *Cancer Epidemiology* 43: 30–34.

Zhao, F. H., Tiggelaar, S. M., Hu, S. Y., Xu, L. N., Hong, Y., Niyazi, M. *et al*. (2012) A multi-center survey of age of sexual debut and sexual behavior in Chinese women: Suggestions for optimal age of human papillomavirus vaccination in China *Cancer Epidemiology* 6(4): 384-390.

Zhao, M., Rosenbaum, E., Carvalho, A. L, Koch, W., Jiang, W., Sidransky, D. *et al*. (2005) Feasibility of quantitative PCR-based saliva rinse screening of HPV for head and neck cancer. *International Journal of Cancer* 117:605–10.